DESLIGNIFICACIÓN ENZIMÁTICA DE MATERIALES LIGNOCELULÓSICOS DE INTERÉS INDUSTRIAL

Memoria que presenta

Alejandro Rico Campos
para optar al título de Doctor en
Ciencias Químicas por la
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Deslignificación enzimática de materiales lignocelulósicos de interés industrial

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DIRECTORES

Dra. Dña. Ana Gutiérrez Suárez
Investigador Científico del CSIC
IRNAS-CSIC

Prof. D. José C. del Río Andrade
Profesor de Investigación del CSIC
IRNAS-CSIC

Dr. D. Jorge Rencoret Pazo
Investigador del CSIC
IRNAS-CSIC

TUTOR

Prof. Dña. Mª Elena Fernández Boy
Profesor Titular de la Universidad de Sevilla

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D. Alejandro Rico Campos
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D. JOSÉ MANUEL PARDO PRIETO, PROFESOR DE INVESTIGACIÓN DEL CSIC Y DIRECTOR DEL INSTITUTO DE RECURSOS NATURALES Y AGROBIOLOGÍA DE SEVILLA DEL CONSEJO SUPERIOR DE INVESTIGACIONES CIENTÍFICAS

Certifica: Que la presente Memoria de Investigación titulada “Deslignificación enzimática de materiales lignocelulósicos de interés industrial”, presentada por D. Alejandro Rico Campos para optar al grado de Doctor en Ciencias Químicas por la Universidad de Sevilla, ha sido realizada en el Departamento de Biotecnología Vegetal, bajo la dirección de la Dra. Dña. Ana Gutiérrez Suárez, el Prof. D. José Carlos del Río Andrade y el Dr. Jorge Rencoret Pazo, reuniendo todas las condiciones exigidas a los trabajos de Tesis Doctorales.

En Sevilla, a 25 de Septiembre de 2014

A mis padres

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ABREVIATURAS

ABTS	2,2′-azino-bis(3-etilbenzotiazolin-6-sulfónico)
AFEX	Explosión amoniacal de la fibra (“Ammonia Fibre Expansion”)
CEL	Lignina aislada con enzimas celulolíticas (“Cellulolytic Enzyme Lignin”)
DMSO-d_6	Dimetilsulfóxido deuterado
DMC	Conversión microbiana directa (“Direct Microbial Conversion”)
2D-NMR o 2D-RMN	Resonancia Magnética Nuclear bidimensional (“Two Dimensional Nuclear Magnetic Resonance”)
3D-NMR o 3D-RMN	Resonancia Magnética Nuclear tridimensional (“Three Dimensional Nuclear Magnetic Resonance”)
ECF	Secuencia de blanqueo libre de cloro elemental (“Elemental Chlorine Free”)
Ep	Extracción alcalina con peróxido
EPA	Agencia de protección medioambiental de EE.UU. (“US Environmental Protection Agency”)
FA	Ácido ferúlico
FPU	Unidades de papel filtro (“Filter Paper Units”)
G	Unidad guayacilpropano (o guayacilo)
GC/MS	Cromatografía de gases/espectrometría de masas (“Gas Chromatography/Mass Spectrometry”)
H	Unidad 4-hidroxifenilpropano (o 4-hidroxifenilo)
HBT	1-Hidroxibenzotriazol
HPLC	Cromatografía líquida de alta resolución (High Performance Liquid Chromatography)
HSQC	Correlación heteronuclear de cuanto simple (“Heteronuclear Single-Quantum Correlation”)
IK	Índice kappa
ISO	Organización internacional de normalización (“International Organization for Standardization”)

LAS	Lignina Ácido-Soluble
LCC	Complejo lignina-carbohidrato (“Lignin-Carbohydrate Complex”)
LiP	Lignina peroxidasa (“Lignin Peroxidase”)
MeS o MS	Metilsiringato
MnP	Manganeso peroxidasa (“Manganese Peroxidase”)
MtL	Lacasa de *Myceliophthora thermophila*
PCA	Ácido *p*-cumárico
PcL	Lacasa de *Pycnoporus cinnabarinus*
Py-GC/MS	Pirólisis acoplada a cromatografía de gases/espectrometría de masas (“Pyrolysis-Gas Chromatography/Mass Spectrometry”)
S	Unidad siringilpropano (o siringilo)
S′	Unidad siringilpropano oxidada
SA	Siringaldehído
SHF	Hidrólisis y fermentación separadas (“Separate Hydrolysis and Fermentation”)
SSF	Hidrólisis y fermentación simultáneas (“Simultaneous Saccharification and Fermentation”)
T20	Tween 20
T80	Tween 80
TAPPI	Asociación técnica de la industria de pasta y papel (“Technical Association of the Pulp and Paper Industry”)
TCF	Secuencia de blanqueo totalmente libre de cloro (“Totally Chlorine Free”)
TMP	Pasta termomecánica (“ThermoMechanical Pulp”)
TvL	Lacasa de *Trametes villosa*
U	Unidad de actividad enzimática
VP	Peroxidasa versátil (“Versatile Peroxidase”)
δ_C	Desplazamiento químico del carbono
δ_H	Desplazamiento químico del protón
ε	Coeficiente de extinción molar

ÍNDICE

RESUMEN

La presente Tesis aborda el estudio de tratamientos enzimáticos (basados en la utilización de lacasas en presencia de mediadores redox) encaminados a eliminar/modificar la lignina y/o lípidos presentes en materiales lignocelulósicos de interés industrial, con el fin de conseguir un aprovechamiento más eficaz y racional de dichos materiales, tanto para la producción de celulosa como de biocombustibles de segunda generación (bioetanol). La importancia de los lípidos en la materia prima radica en que son responsables de la formación de los denominados depósitos de *pitch* durante la fabricación de pasta y papel, reduciendo drásticamente la calidad del producto final y provocando importantes pérdidas económicas. Por otra parte, tanto el contenido como la composición y estructura de la lignina influyen decisivamente en el proceso de deslignificación de la lignocelulosa afectando tanto a los procesos de cocción y blanqueo (en la producción de celulosa) como en la sacarificación enzimática (en la producción de bioetanol).

En esta Tesis se han utilizado lacasas de alto potencial redox, de los hongos basidiomicetos *Trametes villosa* y *Pycnoporus cinnabarinus* y de bajo potencial redox como la lacasa del hongo ascomiceto *Myceliophthora thermophila*. Los tratamientos enzimáticos se realizaron en presencia y ausencia de mediadores redox tanto sintéticos, como el 1-hidroxibenzotriazol (HBT), como fenólicos incluyendo siringaldehído (SA) y siringato de metilo (MeS). Los materiales lignocelulósicos utilizados incluyeron pastas kraft de eucalipto para los estudios de deslignificación/blanqueo y eliminación de lípidos responsables del *pitch*, y madera de eucalipto (*Eucalyptus globulus*) y hierba elefante (*Pennisetum purpureum*) para los estudios de deslignificación como pretratamiento previo a la sacarificación enzimática para la obtención de bioetanol.

En los tratamientos de pasta kraft de eucalipto con la lacasa de *M. thermophila* (MtL) en presencia de los mediadores fenólicos SA y MeS, los mejores resultados de deslignificación (25%) y aumento de blancura (8.3 puntos) de dichas pastas se obtuvieron en el tratamiento con MtL y MeS (seguido de extracción alcalina con peróxido). Sin embargo, la mayor eliminación de los compuestos lipofílicos se consiguió en el tratamiento con MtL en presencia de SA como mediador, produciéndose una disminución del 73% de esteroles libres, 91% de glicósidos de esteroles y del 89% de ésteres de esteroles. Estos tratamientos incluyeron la optimización de las dosis enzima-mediador obteniéndose unos resultados muy prometedores con unas dosis tanto de lacasa como de MeS aceptables para su implementación industrial. Estos resultados fueron la base para la realización de un ensayo de este tratamiento

enzimático a escala piloto que se llevó a cabo en el “Centre Technique du Papier” (Grenoble, Francia).

Otros estudios estuvieron encaminados al desarrollo de pretratamientos enzimáticos basados en la utilización del sistema lacasa-mediador para deslignificar materiales lignocelulósicos procedentes de cultivos de crecimiento rápido (eucalipto y hierba elefante) con el objeto de mejorar el rendimiento de la sacarificación enzimática y la producción de bioetanol. Primeramente se realizaron tratamientos de estos materiales con una lacasa de alto potencial redox (de *T. villosa*) y HBT como mediador, en una secuencia que incluía cuatro tratamientos enzimáticos seguidos cada uno de ellos de una extracción alcalina con peróxido de hidrógeno (8 pasos). Tras la secuencia completa se determinó el contenido en lignina (lignina Klason) y se evaluó el rendimiento de la sacarificación enzimática (con celulasas) y fermentación de las muestras pretratadas. Con estos pretratamientos se consiguió una eliminación de lignina de un 48% en la madera de eucalipto y de un 32% en la hierba elefante, que conllevaron una mejora en el rendimiento de la liberación de glucosa de un 61% y 12% y de producción de etanol de 4 g/L y 2 g/L en la madera de eucalipto y hierba elefante, respectivamente, respecto de las muestras sin pretratamiento enzimático. Además de la variación en el contenido en lignina del material lignocelulósico tras los pretratamientos, se estudió la modificación estructural del polímero de lignina mediante el análisis de las muestras por Resonancia Magnética Nuclear bidimensional (2D-NMR). Estos estudios revelaron una eliminación significativa de las unidades guayacilo (G) y siringilo (S) de la lignina así como de las cadenas laterales (principalmente uniones β-*O*-4’) y una disminución más moderada del contenido en ácido *p*-cumárico (presente en la hierba elefante) sin una variación sustancial de las señales de polisacárido. Estos análisis también mostraron una oxidación en el C_α de las unidades S de la lignina que fue más evidente en el caso de la madera de eucalipto.

Con el fin de facilitar la viabilidad industrial de este pretratamiento enzimático basado en el sistema lacasa-mediador, posteriormente se estudiaron pretratamientos enzimáticos de madera de eucalipto en condiciones similares a los anteriormente descritos, pero utilizando un sistema lacasa-mediador de más bajo coste, que consistió en la utilización de una lacasa comercial (MtL) y de un mediador fenólico (MeS). Tras el pretratamiento completo (cuatro ciclos) se obtuvo una eliminación del 50% del contenido en lignina de la madera de eucalipto así como un aumento de un 40% en la liberación de glucosa tras sacarificación enzimática, respecto de las muestras sin pretratamiento enzimático. La modificación estructural de la lignina producida por estos pretratamientos se estudió en profundidad mediante 2D-NMR. Estos análisis revelaron una eliminación significativa de las unidades guayacilo

(G) y siringilo (S) de la lignina con preferencia de las primeras, así como de las cadenas laterales (principalmente uniones β-*O*-4' y β-β'). La modificación más relevante observada fue la oxidación en el C_α de las unidades S. En estos pretratamientos se llevó a cabo un estudio detallado por 2D-NMR de la modificación estructural de la lignina tras cada uno de los 8 pasos (incluyendo tratamientos enzimáticos y extracción alcalina) tanto en el material lignocelulósico completo como en las ligninas aisladas enzimáticamente y en los filtrados de los tratamientos, lo que ha aportado información de gran interés sobre el mecanismo de degradación de la lignina por el sistema lacasa-mediador y que será de ayuda para desarrollar pretratamientos enzimáticos viables industrialmente.

La presente Tesis incluye los siguientes apartados: I) una introducción general sobre los cultivos lignocelulósicos, su composición, su interés industrial, y los procesos utilizados tanto para la producción de pasta de celulosa como para la producción de biocombustible de segunda generación, así como los principales problemas que plantean algunos de sus constituyentes y algunas soluciones biotecnológicas a estos problemas; II) los objetivos perseguidos en la Tesis; III) una descripción detallada de los materiales estudiados y los métodos analíticos empleados; IV) los resultados generales obtenidos y su discusión; V) las referencias citadas; VI) las publicaciones científicas; y VII) las principales conclusiones.

ABSTRACT

The present Thesis deals with the development of enzymatic treatments (based on the use of laccases in the presence of redox mediators) aimed at the removal/modification of the lignin and/or lipids present in lignocellulosic materials of industrial interest, in order to achieve a more efficient and rational use of these materials for the production of both, cellulose and second generation biofuels (bioethanol). The importance of lipids in the raw materials is because they are responsible for the formation of the so-called pitch deposits during pulp and paper manufacturing, drastically reducing the quality of the final product and producing important economic losses. Moreover, the content, composition and structure of the lignin have a significant effect on the delignification process of the lignocellulose, affecting both the cooking and bleaching processes (during the production of cellulose) or the enzymatic saccharification (during the production of bioethanol).

In this Thesis, high redox potential laccases from the fungal basidiomycetes *Trametes villosa* and *Pycnoporus cinnabarinus* as well as a low redox potential laccase from the fungal ascomycete *Myceliophthora thermophila* have been used. The enzymatic treatments were performed in the presence and absence of redox mediators, including synthetic compounds such as 1-hidroxybenzotriazole (HBT), and natural phenolic compounds such as syringaldehyde (SA) and methyl syringate (MeS). The lignocellulosic materials used included eucalyptus kraft pulps for the studies regarding delignification/bleaching and removal of lipids responsible for pitch, and eucalyptus wood (*Eucalyptus globulus*) and elephant grass (*Pennisetum purpureum*) for studies about enzymatic delignification as pretreatment prior enzymatic saccharification for the production of second generation bioethanol.

In the enzymatic treatments of eucalyptus kraft pulps with the laccase from *M. thermophila* (MtL) in the presence of the phenolic mediators SA and MeS, the best results of delignification (25%) and whiteness increase (8.3 points) were obtained with MtL and MeS (followed by alkaline peroxide extraction). However, the highest removal of the lipophilic compounds were obtained using MtL in the presence of SA as mediator, resulting in a decrease of 73% of free sterols, 91% of sterol glycosides and 89% of sterol esters. These treatments included the optimization of

enzyme-mediator doses, obtaining very promising results with doses of MtL and MeS low enough for further industrial implementation. These results were the basis for additional scale-up of the enzymatic treatment at pilot scale, that was carried out at the “Centre Technique du Papier” (Grenoble, France).

Other studies were aimed at the development of enzymatic pretreatments based on the use of the laccase-mediator system for the delignification of lignocellulosic materials from fast growing crops (eucalyptus and elephant grass) in order to improve the yields of the enzymatic saccharification and the production of bioethanol. The pretreatments of these materials were initially performed with a high redox potential laccase (from *T. villosa*) and HBT as mediator in a multistage sequence consisting of successive enzymatic and alkaline peroxide stages. After completing the sequence, the lignin content (Klason lignin) was determined and the yields of the enzymatic saccharification (using cellulases) and fermentation of the pretreated samples were evaluated. These pretreatments removed up to 48% of the lignin in eucalyptus and up to 32% of the lignin in elephant grass, leading to an improvement in the glucose yield of 61% and 12%, and ethanol yield of 4 g/L and 2 g/L in eucalyptus wood and elephant grass, respectively, with respect to the control samples without pretreatment. Besides the variation in the lignin content of the lignocellulosic material after the pretreatments, the structural modification of the lignin polymer was studied by two-dimensional nuclear magnetic resonance (2D-NMR). These studies revealed a significant removal of guaiacyl (G) and syringyl (S) lignin units and aliphatic side chains (mainly β-*O*-4′ linkages) and a more moderate decline of the *p*-coumaric acid content (present in elephant grass) without a substantial change in the polysaccharide signals. The analyses also indicated a Cα oxidation of the S-lignin units that was more evident in the case of eucalyptus wood.

In order to facilitate the potential industrial feasibility of the enzymatic pretreatments based on the laccase-mediator system, the enzymatic pretreatment of eucalyptus wood was studied under similar conditions as described above but using a low cost laccase-mediator system, constituted by a commercial laccase (MtL) and a natural phenolic mediator (MeS). This pretreatment (four cycles) resulted in a 50% lignin removal in the eucalyptus wood and a 40% increase in glucose yields after the enzymatic saccharification. The structural changes occurring in the lignin during these pretreatments were studied in depth by 2D-NMR.

The analyses revealed a significant removal of guaiacyl (G) and syringyl (S) lignin units, with a preferential removal of the former, as well as of the side chains (mainly β-*O*-4′ and β-β′ linkages). The most significant modification observed was the oxidation of S-lignin units at Cα. Furthermore, a detailed 2D-NMR study of the structural modifications of the lignin was carried out after each of the 8 steps (including the enzymatic treatments and the alkaline extraction) in both, the whole cell walls and in isolated lignins, as well as in the filtrates, that has provided information of great interest about the mechanism of lignin degradation by the laccase-mediator system that will help to develop industrially feasible enzymatic pretreatments.

The present Thesis includes the following sections: I) a general introduction about lignocellulosic materials, their composition, their industrial interests, the processes used for the production of cellulose pulp and second generation biofuel, as well as the main problems originated by their constituents and some biotechnological solutions to address them; II) the objectives pursued in the Thesis; III) a detailed description of the selected materials and the analytical methods used; IV) the overall results obtained and their discussion; V) the cited references; VI) the scientific publications; and VII) the main conclusions.

I.1. BIOMASA LIGNOCELULÓSICA

La biomasa lignocelulósica representa la mayor fuente de energía y material renovable de la Tierra, y tiene por tanto un gran potencial como materia prima para la producción de celulosa, bioenergía y otros productos de interés industrial. La principal fuente de biomasa lignocelulósica la constituyen los cultivos forestales y agrícolas, aunque los residuos generados a partir de estos cultivos también presentan interés como materia prima.

I.1.1. Cultivos lignocelulósicos

Los **cultivos forestales** (**Figura 1**) incluyen especies de coníferas y frondosas. Ambas especies se utilizan a escala mundial para la obtención de pasta de papel. En términos económicos generales, las coníferas son más valiosas que las frondosas, ya que sus troncos son más largos y rectos, su madera es uniforme, ligera y blanda, presentan fibras largas (entre 3 y 5 mm) que ofrecen una alta resistencia mecánica, y son más adecuadas para la mayoría de las calidades papeleras (García Hortal 2007). Las principales coníferas utilizadas para la fabricación de pasta de papel son la *Picea* y el pino. La madera de frondosas, por otro lado, es una madera más dura, de fibras cortas (entre 0.75 y 2 mm) que dan lugar a pastas menos uniformes. El papel fabricado con maderas de frondosas es más débil que los fabricados con maderas de coníferas pero su superficie es más lisa, y por lo tanto, es mejor para papel de escritura. Otra de las ventajas es que el crecimiento de las especies de frondosas utilizadas para la fabricación de pasta de papel es más rápido que el de las coníferas, dando lugar a mayor cantidad de fibra en menos tiempo. Así, los cultivos madereros de crecimiento rápido son atractivos para la industria papelera como también lo son para la obtención de bioetanol. Las principales especies de frondosas utilizadas en el sector papelero y potencialmente interesantes para la producción de bioetanol son el eucalipto, el chopo y el abedul.

Los **cultivos agrícolas** (**Figura 1**) constituyen una excelente materia prima alternativa a los cultivos forestales para la obtención de celulosa, principalmente por su gran abundancia y su coste relativamente bajo, especialmente en países con baja disponibilidad de madera. Presentan ciclos de crecimiento más cortos, alcanzando la madurez más rápidamente que las especies madereras. El principal inconveniente de este tipo de materias primas es que la mayoría sólo están disponibles en ciertas épocas del año. Ejemplos de cultivos no madereros son: hierba elefante, bambú, cáñamo, lino, kenaf, yute, etc.

Por otro lado, los residuos generados de los cultivos agrícolas, como el rastrojo de maíz, la paja de trigo, de arroz y de otros cereales,

constituyen también una fuente importante de material lignocelulósico (Kim y Dale 2004).

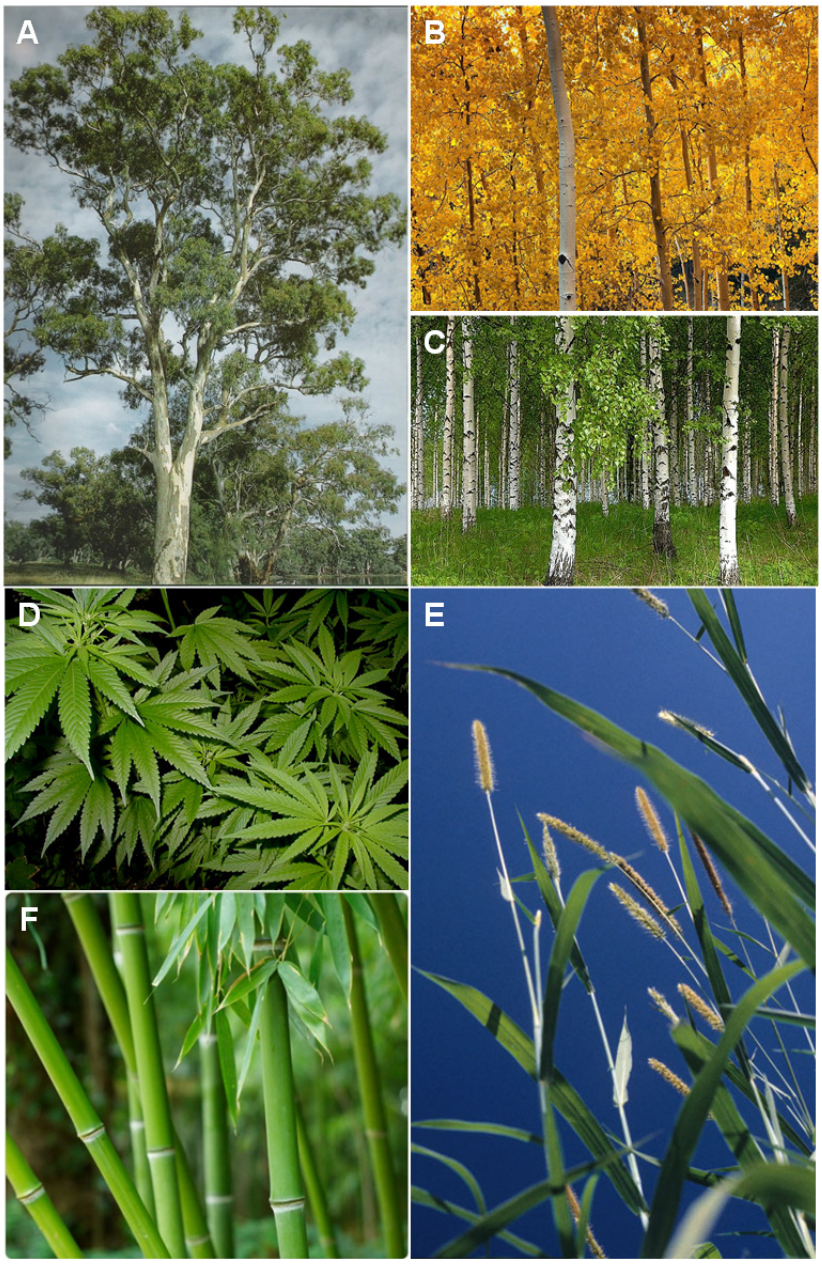


Figura 1. Fotografía de diferentes especies de cultivos forestales: (A) eucaliptos, (B) álamos, (C) abedules; y cultivos agrícolas: (D) cáñamo, (E) hierba elefante y (F) bambú.

I.1.2. Composición de los materiales lignocelulósicos

Los materiales lignocelulósicos están formados por tres polímeros estructurales mayoritarios, que constituyen las paredes celulares de los materiales vegetales: celulosa (40-50%), hemicelulosas (20-30%) y lignina (10-30%); y una serie de compuestos minoritarios de bajo peso molecular solubles en agua o en solventes orgánicos, así como pequeñas cantidades de proteínas y sales minerales (Fengel y Wegener 1984; Sjöström 1993).

La pared celular vegetal (**Figura 2**) está constituida por varias capas: pared primaria (P), pared secundaria externa (S_1), pared secundaria media (S_2), y pared secundaria interna (S_3). Rodeándolas se encuentra la lámina media, compuesta principalmente de sustancias pécticas al inicio de la fase de crecimiento, pero que en la fase de maduración está muy lignificada.

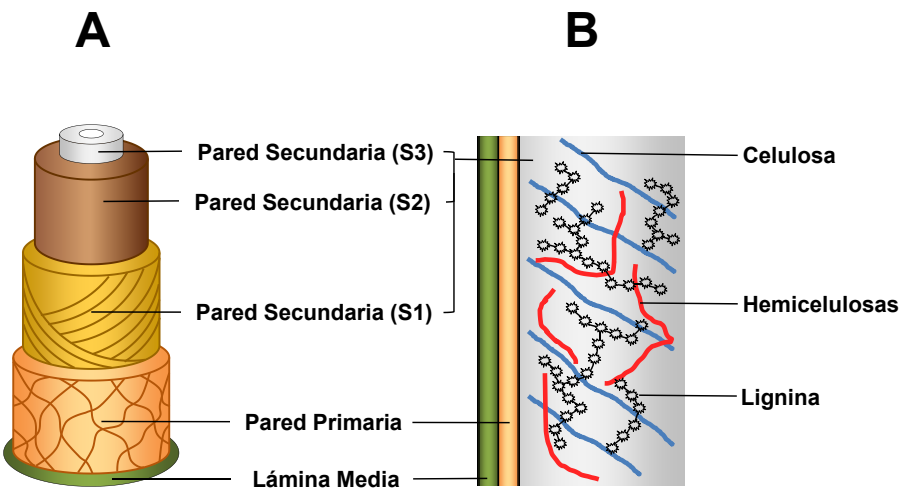


Figura 2. (A) Organización estructural de la pared celular vegetal y (B) distribución de los distintos componentes.

Celulosa

La celulosa es el polímero orgánico más abundante en la Tierra (Klemm *et al.* 2005). Presenta una estructura lineal, altamente ordenada, formada por unidades de β-D-glucopiranosa unidas entre sí por enlaces glicosídicos β (1→4) (**Figura 3**). Durante la formación de este enlace, la posición β de los grupos hidroxilos en el C1 necesita un giro de 180º de

la siguiente molécula de glucosa, por lo que la unidad que se repite es la celobiosa, un disacárido que representa la unidad constitutiva de la celulosa (Fengel y Wegener 1984; Sjöstrom 1993)

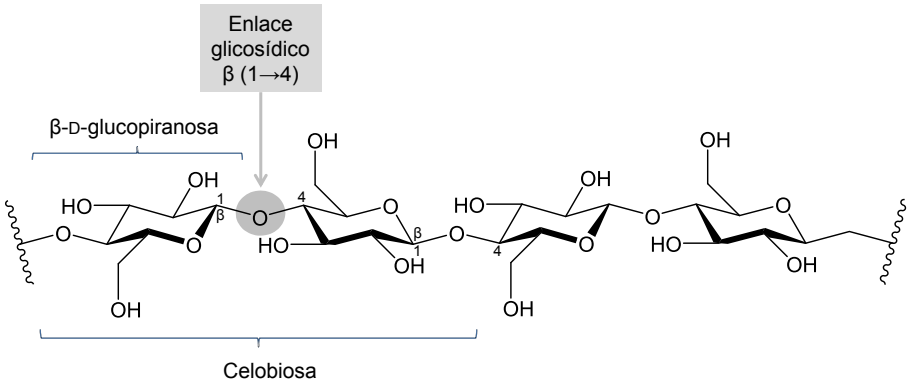


Figura 3. Estructura de una cadena de celulosa.

Las cadenas de celulosa son completamente lineales y tienden a formar puentes de hidrógeno intra e intermoleculares. Estas uniones con otras cadenas que están en el mismo plano, así como con cadenas en planos superiores e inferiores, dan lugar a la formación de microfibrillas. La unión de las microfibrillas da lugar a la formación de macrofibrillas, y la unión de éstas entre sí a la fibra de celulosa (**Figura 4**).

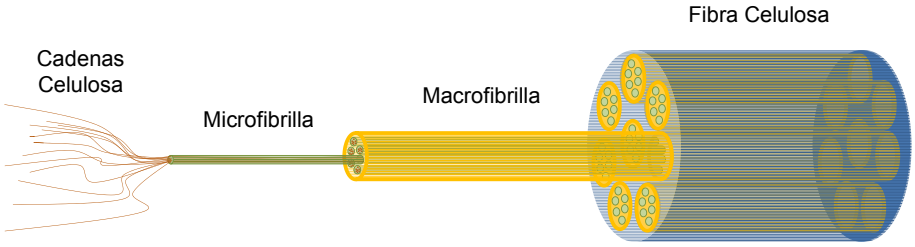


Figura 4. Estructuración de cadenas de celulosa en micro- y macrofibrillas.

Hemicelulosas

Son polisacáridos químicamente heterogéneos, constituidos por diferentes unidades de monosacáridos incluyendo pentosas (D-xilosa y L-arabinosa), hexosas (D-glucosa, D-galactosa, L-galactosa, D-manosa, L-ramnosa y L-fucosa) y ácidos urónicos (ácido D-glucurónico y ácido D-galacturónico) (**Figura 5**), unidos entre sí por enlaces glicosídicos β(1→4), y en algunos casos β(1→3), formando estructuras ramificadas y en general amorfas.

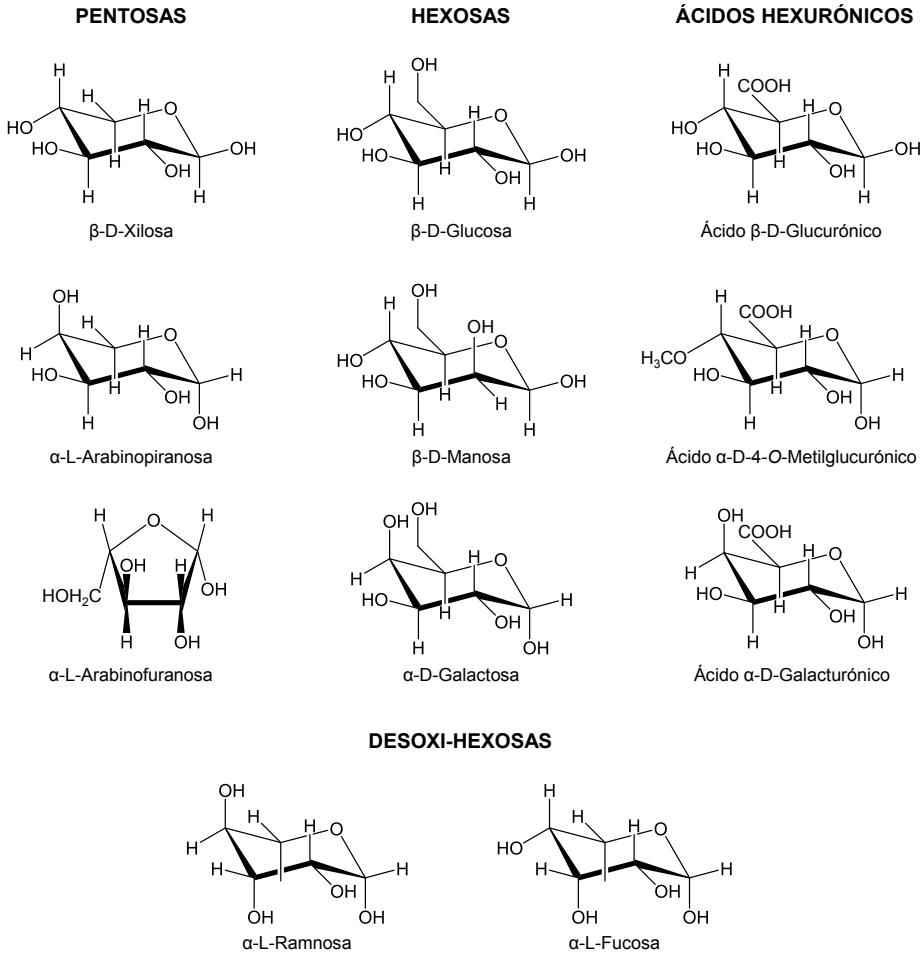


Figura 5. Monosacáridos componentes de las hemicelulosas (Fengel y Wegener 1984).

Algunas hemicelulosas están asociadas a la porción celulósica, mientras que otras lo están a la lignina. Actúan como matriz soporte para las microfibrillas de celulosa en la pared celular, y son de menor masa molecular, más accesibles, más fácilmente degradables y más fáciles de disolver que la celulosa.

Lignina

La lignina es el constituyente vegetal más recalcitrante y, después de la celulosa, el polímero más abundante en la superficie de la Tierra. Este componente de la pared celular vegetal realiza múltiples funciones que son esenciales para la vida de las plantas, entre las que destacan su importante papel en el transporte interno de agua, nutrientes y metabolitos, el proporcionar rigidez a la pared celular y actuar como puente de unión entre las células de la madera, creando un material que es notablemente resistente a los impactos, compresiones y flexiones (Chabannes *et al.* 2001; Jones *et al.* 2001). Además, juega un papel importante en la defensa de la planta frente a patógenos (Sarkanen y Ludwig 1971).

La lignina, a diferencia de la celulosa y de las hemicelulosas, presenta una estructura muy compleja, ya que se trata de un polímero aromático muy ramificado y amorfo. Presenta una estructura muy compleja sintetizada a partir de la fenilalanina (**Figura 6**) a través de la ruta de los ácidos cinámicos (Higuchi 1997; Boerjan *et al.* 2003). De esta forma se sintetizan de manera preferente tres compuestos que actúan como precursores de la lignina, los monolignoles alcohol *p*-cumarílico (4-hidroxicinamílico), alcohol coniferílico (4-hidroxi-3-metoxicinamílico) y alcohol sinapílico (4-hidroxi-3,5-dimetoxicinamílico). Estos tres monolignoles principales difieren entre sí en el número de grupos metoxilo que contienen en el anillo aromático. Cuando estos alcoholes se incorporan a la lignina dan lugar a las unidades *p*-hidroxifenilo (unidad H), guayacilo (unidad G) y siringilo (unidad S), respectivamente.

Además de los tres precursores principales de la lignina (alcohol *p*-cumarílico, alcohol coniferílico y alcohol sinapílico), recientemente, y debido fundamentalmente a los avances en el campo de la Resonancia Magnética Nuclear bidimensional (2D-NMR), se han descubierto otros compuestos que pueden participar como precursores de la lignina (**Figura 7**). Entre ellos destacan:

- Derivados γ-acilados de los correspondientes alcoholes *p*-hidroxicinamílicos. Entre los principales grupos acilantes descubiertos hasta la fecha cabe destacar: acetatos (**A**) (del Río *et al.* 2007), *p*-cumaratos (**B**) (del Río *et al.* 2008) y benzoatos (**C**) (Rencoret *et al.* 2013).

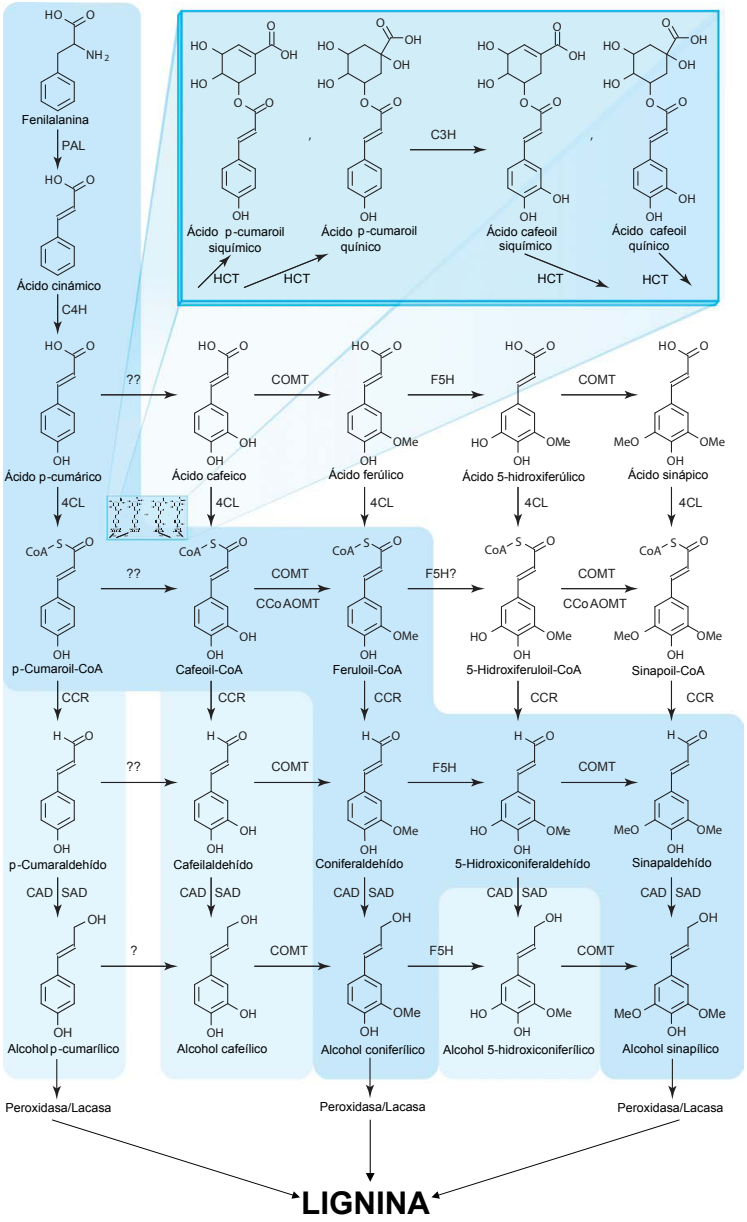


Figura 6. Ruta biosintética de la lignina en angiospermas (Boerjan *et al.* 2003). La ruta en azul es la más favorecida. Las enzimas que intervienen en esta ruta biosintética son las siguientes: fenilalanina amonio liasa (PAL), cinamato-4-hidroxilasa (C4H), 4-cumarato CoA ligasa (4CL), cafeico *O*-metiltransferasa (COMT), cinamoil CoA-reductasa (CCR), cafeoil CoA-3-*O*-metiltransferasa (CCoAOMT), cinamil alcohol deshidrogenasa (CAD), ferulato-5-hidroxilasa (F5H), sinapil alcohol deshidrogenasa (SAD), *p*-cumarato-3-hidroxilasa (C3H).

- Compuestos intermediarios de la ruta biosintética de la lignina, tales como aldehídos cinamílicos (**D**) (Kim *et al.* 2003), ácido ferúlico (**E**) (Leplé *et al.* 2007), alcohol cafeílico (**F**) (Chen *et al.* 2012) y alcohol 5-hidroxiconiferílico (**G**) (Chen *et al.* 2013).
- Compuestos no relacionados con la ruta biosintética de la lignina. Compuestos que pertenecen a otra ruta biosintética, similares a los monolignoles, y que se encuentran en el lugar de lignificación, y terminan incorporándose a la lignina, como ocurre con la tricina (**H**) en la paja de trigo (del Río *et al.* 2012).

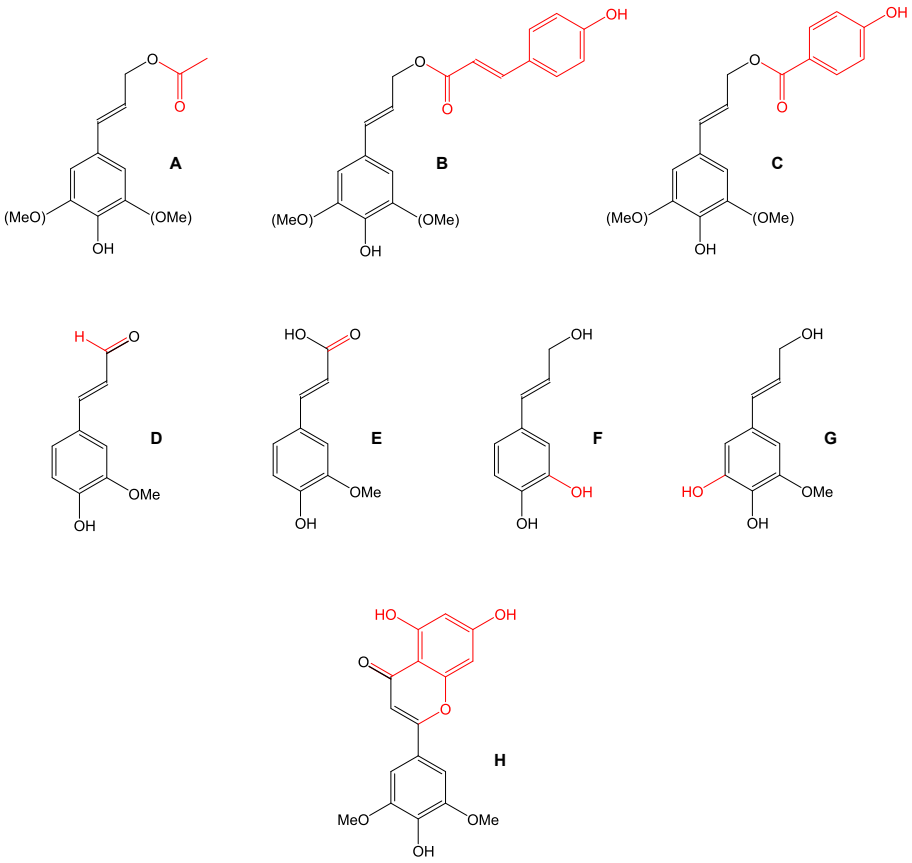


Figura 7. Estructuras de precursores de la lignina: (A) acetato, (B) *p*-cumarato, (C) benzoato, (D) aldehído correspondiente a los alcoholes *p*-hidroxicinamílicos, (E) ácido ferúlico, (F) alcohol cafeílico, (G) alcohol 5-hidroxiconiferílico y (H) tricina.

La formación del polímero de lignina (lignificación) comienza con la deshidrogenación enzimática de los monolignoles para formar radicales libres de tipo fenoxilo, estabilizados por resonancia (**Figura 8**). La deshidrogenación enzimática consiste en una reacción de transferencia de un electrón catalizada por peroxidasas en presencia de peróxido de hidrógeno o por lacasas en presencia de oxígeno (Freudenberg y Neish 1968; Adler 1977; Baucher *et al.* 1998; Boerjan *et al.* 2003; Ralph *et al.* 2004).

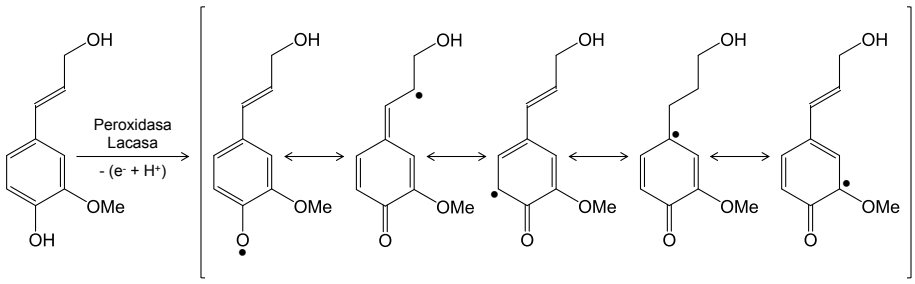


Figura 8. Deshidrogenación del alcohol coniferílico y formas resonantes del radical fenoxilo (adaptado de Adler 1977).

A continuación, tiene lugar el acoplamiento (**Figura 9**) de estos monolignoles radicales entre sí y con el polímero creciente de lignina mediante diversos tipos de uniones.

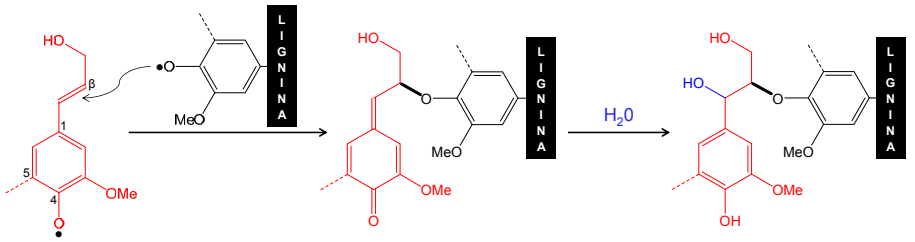


Figura 9. Mecanismo de la unión de los monolignoles libres al polímero de lignina (Freudenberg y Neish 1968).

Aunque la variedad de uniones entre monolignoles y de monolignoles con el polímero creciente de lignina es amplia (**Figura 10**), se pueden diferenciar dos tipos de enlaces: de tipo éter y de tipo carbono-carbono.

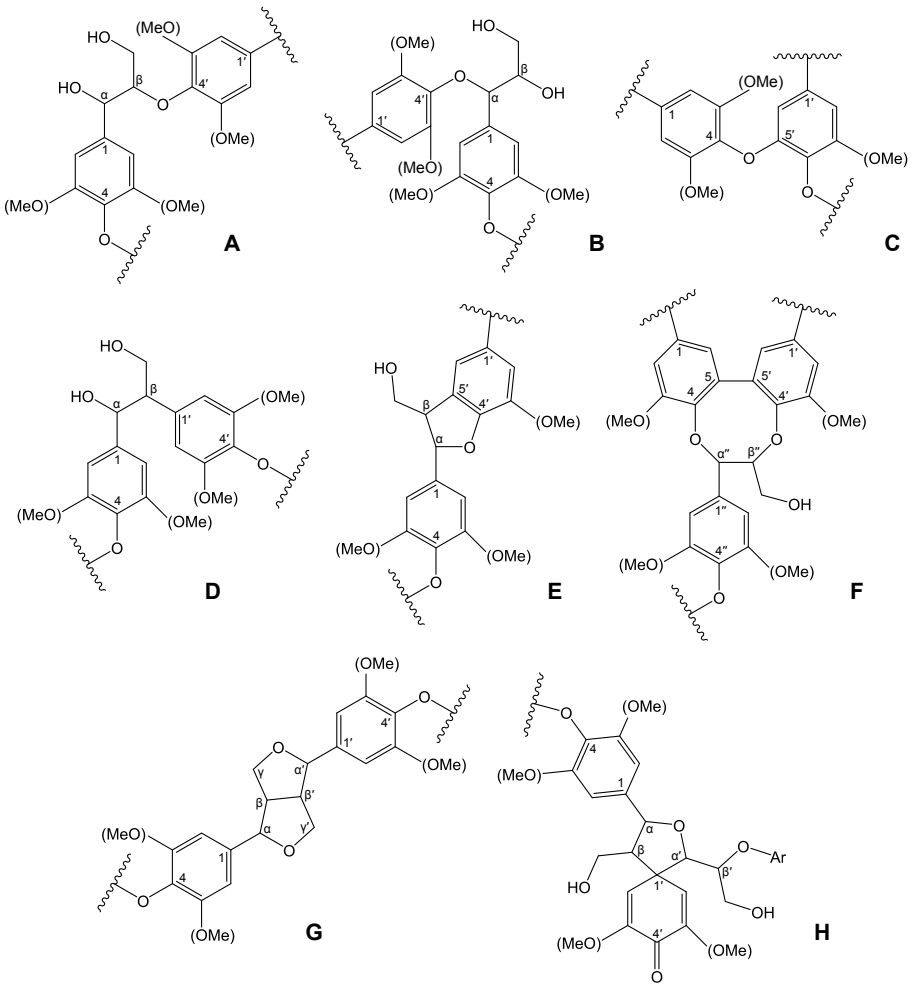


Figura 10. Enlaces tipo éter y carbono-carbono presentes en el polímero de lignina: (A) β-*O*-4′, (B) α-*O*-4′, (C) 4-*O*-5′, (D) β-1′, (E) β-5′/α-*O*-4′ fenilcumarano, (F) 5-5′/α″-*O*-4/β″-*O*-4′ dibenzodioxocina, (G) β-β′/α-*O*-γ′/γ-*O*-α′ resinol y (H) β-1′/α-*O*-α′ espirodienona.

Entre las uniones de tipo éter se diferencian principalmente dos tipos, la unión alquil-aril éter (la más estable termodinámicamente), siendo la unión más corriente de este tipo el enlace β-*O*-4′ (**A**), mientras que el enlace α-*O*-4′ (**B**) es menos frecuente. Por otro lado, en menor proporción existe la unión aril-aril éter, como por ejemplo la unión 4-*O*-5′ (**C**). Las uniones o enlaces de tipo carbono-carbono son también conocidas como enlaces condensados. Son más difíciles de romper que los enlaces de tipo éter e incluyen las uniones de dos cadenas alifáticas

(β-β′ resinol) (**G**), la unión de un carbono de un anillo bencénico con el de una cadena alifática de otra unidad, como los enlaces β-1′ (**D**) y β-5′ fenilcumarano (**E**) y las uniones entre carbonos de anillos bencénicos (5-5′ o bifenilo). Se ha descrito que el enlace 5-5′ no se encuentra tal cual, sino en forma de trímero, ya que incorpora una nueva unidad mediante un enlace β″-*O*-4′ y un enlace α″-*O*-4, dando lugar a una estructura de tipo dibenzodioxocina (**F**) (Karhunen *et al*. 1995). Igualmente, las uniones β-1′ se encuentran preferentemente en forma de espirodienonas (**H**) (Zhang y Gellerstedt 2001; Zhang *et al.* 2006).

La composición de la lignina depende de su origen botánico: la lignina de coníferas presenta principalmente unidades G, la lignina de frondosas unidades G y S en diversas proporciones y la lignina de plantas herbáceas contiene unidades H, G y S en diversas proporciones. Incluso en un mismo árbol, la composición de la lignina puede variar dependiendo de la edad (Freudenberg y Lehmann 1963; Rencoret *et al*. 2011), la parte del árbol (Bland 1966), el tipo de células (Fergus y Goring 1970; Hardell *et al.* 1980a, 1980b) y del lugar de la pared celular o la lámina media donde se sintetice (Fergus y Goring 1970; Fukushima y Terashima 1991; Christiernin *et al.* 2005).

Las unidades G tienen un único grupo metoxilo y la posición C-5 libre y disponible para la formación de enlaces carbono-carbono, por lo que las ligninas con mayor cantidad de unidades G tienen una estructura más condensada y por lo tanto la lignina se degrada con mayor dificultad (del Río *et al.* 2005) que las ligninas con un mayor contenido en unidades S. Por esta misma razón, debido a no presentar ningún grupo metoxilo y tener la posición C-3 y C-5 libres, las unidades H son las que dan lugar a una lignina con mayor grado de condensación. Curiosamente, en plantas transgénicas con unidades H mayoritariamente, se obtiene mayor liberación de glucosa que en plantas naturales con una cantidad mínima de unidades H con respecto a las unidades S y G (Bonawitz *et al.* 2014).

La estructura del polímero de lignina no se conoce con exactitud, y hasta el momento tan sólo se han descrito modelos estructurales. Los primeros modelos estructurales de lignina de coníferas y frondosas datan de los años setenta (Nimz 1974; Adler 1977) y con el paso del tiempo se han ido mejorando gracias al avance de las técnicas analíticas, especialmente de la 2D-NMR, que ha permitido el descubrimiento de nuevas subestructuras. Los modelos más actuales de la estructura de la lignina de coníferas y frondosas se muestran en la **Figura 11**, donde se puede observar la presencia de las nuevas unidades descubiertas en los últimos años, tales como dibenzodioxocinas (Karhunen *et al.* 1995) y espirodienonas (Zhang y Gellerstedt 2001; Zhang *et al.* 2006).

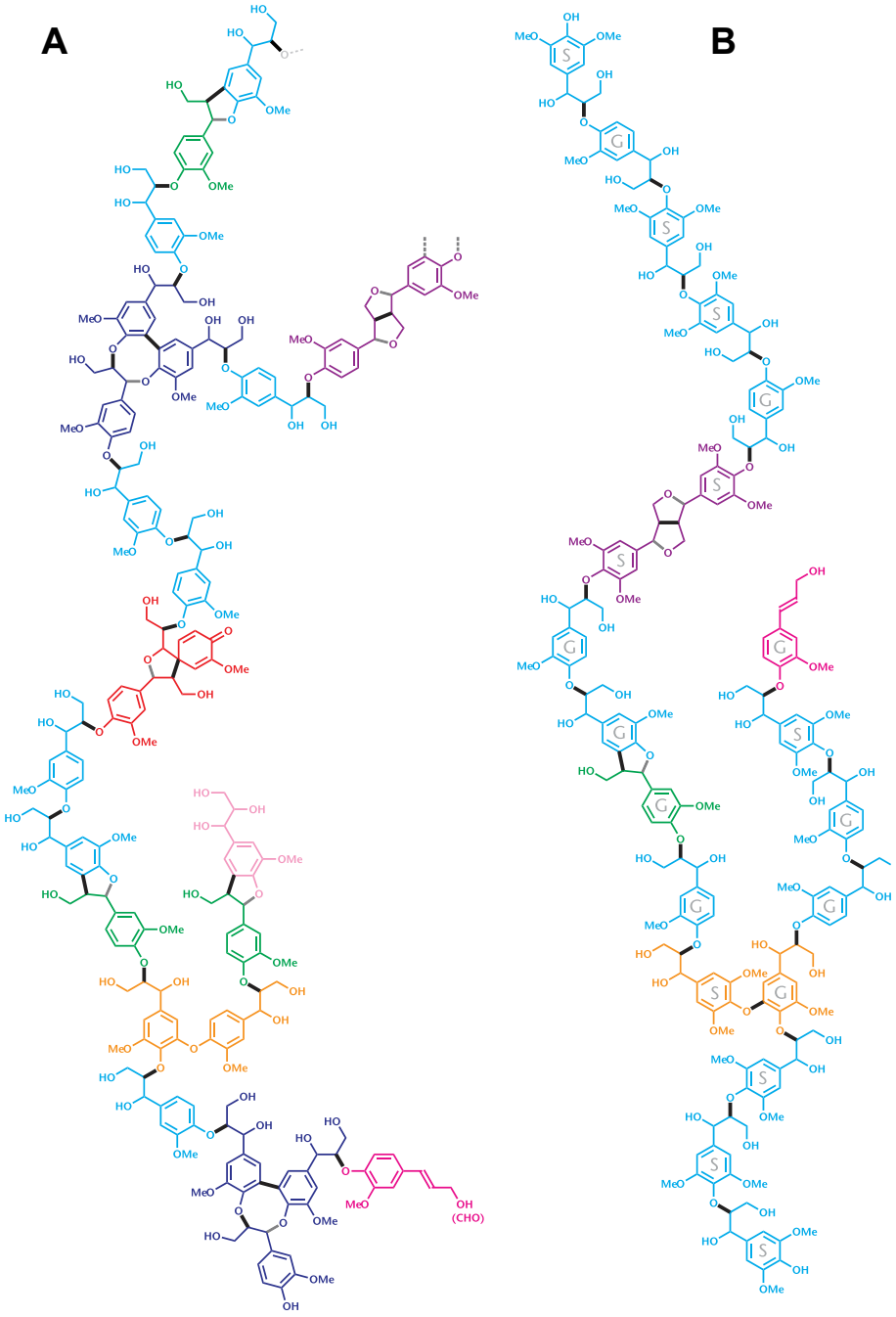


Figura 11. Modelos del polímero de lignina en maderas: (A) conífera (*Picea*) (Brunow 2001) y (B) frondosa (álamo) (Boerjan *et al*. 2003).

La lignina se encuentra unida covalentemente a los carbohidratos, formando los llamados complejos lignina-carbohidrato (LCC) (Watanabe 2003). En general se acepta que existen tres tipos de uniones lignina-carbohidrato, glucósidos de fenilo, ésteres del ácido 4-*O*-metilglucurónico con el C_γ de la lignina y éteres de bencilo (Fengel y Wegener 1984) (**Figura 12**).

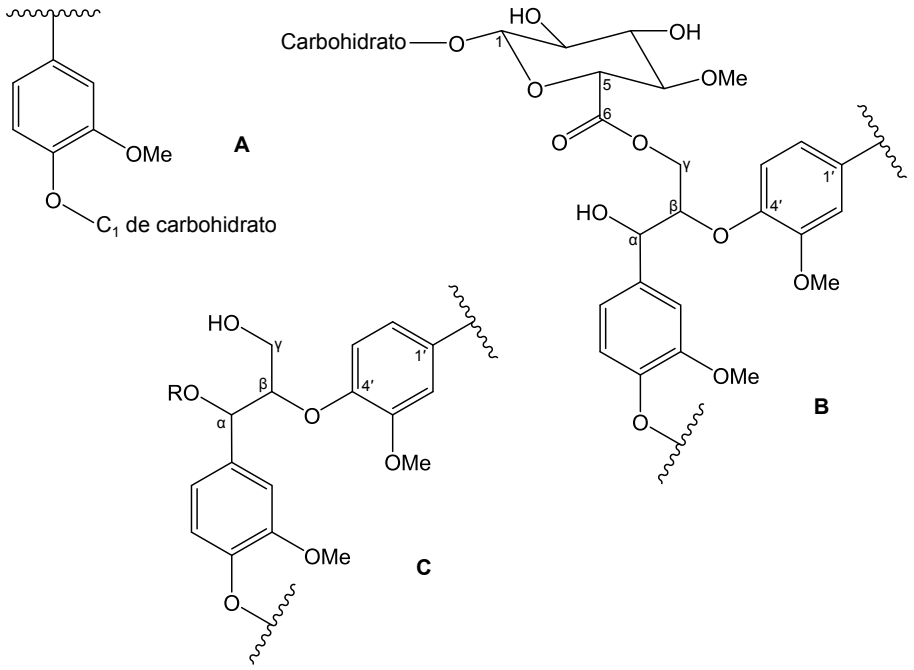


Figura 12. Tipos de uniones lignina-carbohidrato: (A) glucósido de fenilo, (B) éster de ácido 4-*O*-metilglucurónico con el Cγ de la lignina y (C) éter de bencilo, diferentes unidades de azúcar unidas con el C_α de la lignina (R= C6 en la glucosa, manosa, galactosa o C5 en arabinosa). Adaptado de Balakshin *et al.* (2011).

Compuestos minoritarios

Además de los carbohidratos (celulosa y hemicelulosas) y lignina, existen en los materiales lignocelulósicos otros componentes presentes en pequeñas cantidades que pueden tener importancia cuando estos materiales se utilizan como materia prima en la producción de pasta y papel. Algunos de estos compuestos protegen a la madera de los insectos y son responsables de su color, olor y gusto. Se pueden dividir en extraíbles lipofílicos e hidrofílicos y compuestos insolubles.

Los extraíbles lipofílicos se pueden extraer del material lignocelulósico con disolventes apolares. Los extraíbles lipofílicos (**Figura 13**) incluyen: alcanos (**A**), alcoholes grasos (**B**), aldehídos (**C**), ácidos grasos (**D**), esteroles (**E**), ácidos resínicos (**F**), ceras (ésteres de ácidos grasos con alcoholes de cadena larga (**G**)) y glicéridos (ésteres de ácidos grasos con glicerol (**H**)). Los esteroles pueden estar formando glicósidos y acilglicósidos (Gutiérrez y del Río 2001b), siendo el más abundante el sitosterol 3β-D-glucopiranósido (**I**), y también pueden encontrarse esteroles libres o esterificados con ácidos grasos (ésteres de esteroles (**J**)).

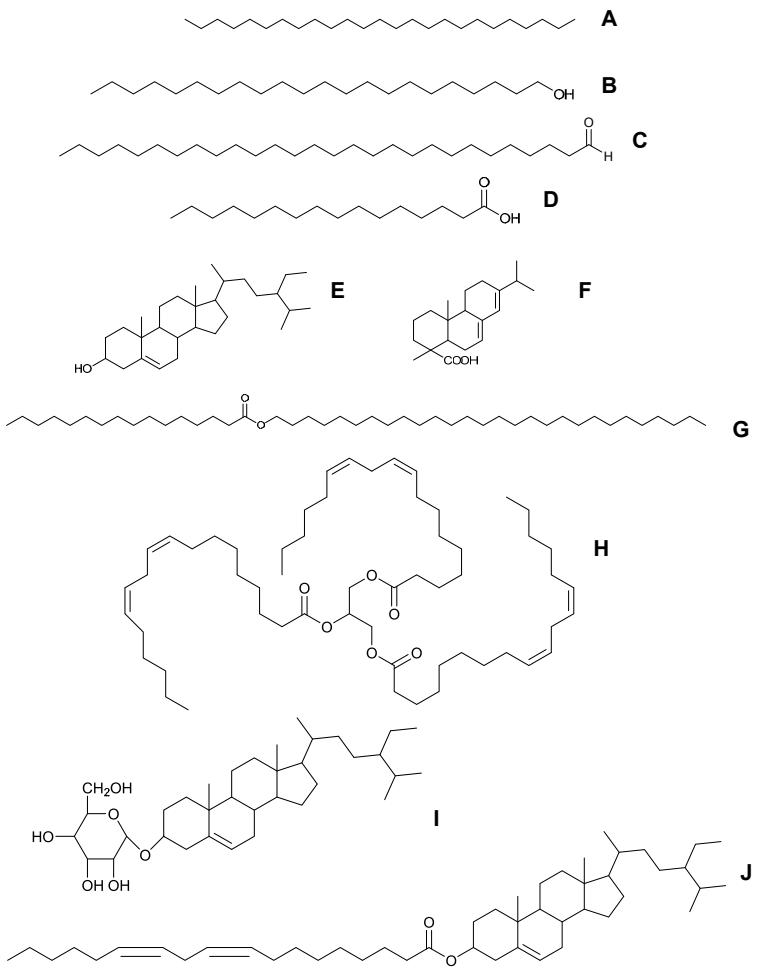


Figura 13. Estructuras de compuestos representativos de las principales familias de extraíbles lipofílicos: (A) *n*-pentacosano, (B) *n*-docosanol, (C) *n*-octacosanol, (D) ácido palmítico, (E) sitosterol, (F) ácido abiético, (G) octacosanil hexadecanoato, (H) trilinoleína, (I) sitosterol 3β-D-glucopiranósido y (J) sitosteril linoleato.

Los extraíbles hidrofílicos se pueden aislar del material lignocelulósico mediante extracciones con disolventes polares. Los extraíbles hidrofílicos (**Figura 14**) incluyen compuestos fenólicos de bajo peso molecular tales como precursores de la lignina (ácidos *p*-hidroxicinámicos y aldehídos *p*-hidroxicinamílicos), ácidos bencenocarboxílicos relacionados (ácido *p*-hidroxibenzoico, vainíllico y siríngico), aldehídos y cetonas aromáticas (*p*-hidroxibenzaldehído, vainillina, siringaldehído y propioguayacona), lignanos (dilignoles y compuestos relacionados), taninos hidrolizables (ésteres del ácido gálico y sus dímeros), taninos no hidrolizables (varias unidades de flavonoides condensadas) y flavonoides (estructuras derivadas del anillo de flavona, 2-fenilbenzopirona).

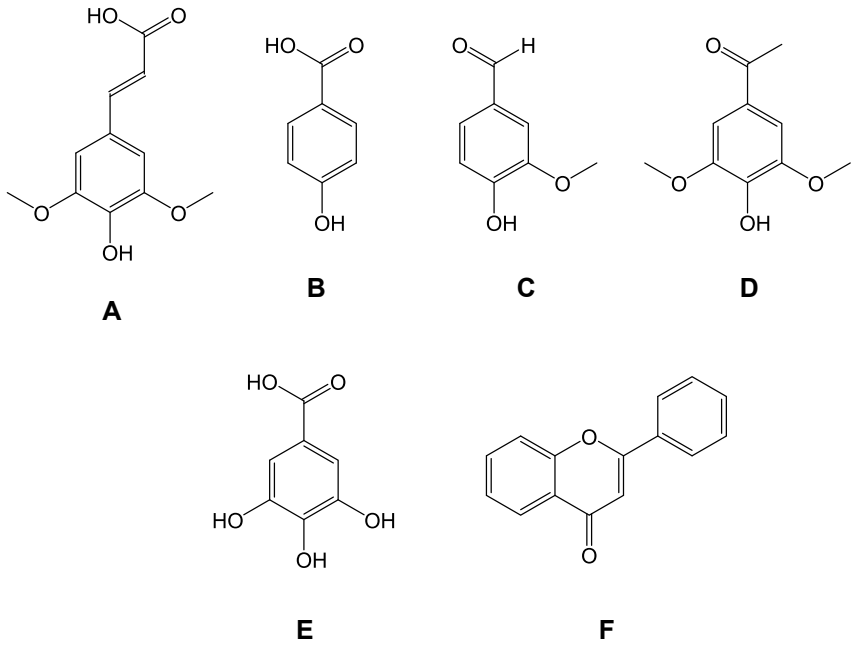


Figura 14. Estructuras de compuestos representativos de los compuestos extraíbles polares: (A) ácido siríngico, (B) ácido *p*-hidroxibenzoico, (C) vainillina, (D) acetosiringona, (E) ácido gálico y (F) 2-fenilbenzopirona.

Los compuestos insolubles son sustancias proteicas, pécticas y de naturaleza inorgánica (García Hortal 2007; Hillis 1962; Fengel y Wegener 1984; Rowe 1989; Sjöström 1993) y su contenido varía con el origen botánico, y en general, es más elevado en las plantas herbáceas (Lu y Ralph 2010).

I.2. BIORREFINERÍA DE LA LIGNOCELULOSA

El término biorrefinería se puede atribuir a la utilización eficiente e íntegra de todos los componentes de la biomasa (residuo cero). Además tiene por objeto aumentar la sostenibilidad tanto a nivel de uso de recursos naturales, a nivel técnico-económico, como a nivel medioambiental. Mediante una serie de procesos químicos, físicos y/o biológicos, la biomasa se transforma para dar lugar a una amplia variedad de productos, como productos energéticos, biopolímeros y productos químicos de base biológica. Estos productos pueden ser utilizados por la propia biorrefinería.

La biorrefinería de material lignocelulósico (**Figura 15**) consiste en la utilización de todos los componentes de la lignocelulosa (celulosa, hemicelulosas y lignina). El alto grado de entrecruzamiento que existe entre los constituyentes lignocelulósicos es una de las principales barreras para el desarrollo de esta biorrefinería, tanto por la resistencia de la matriz lignocelulósica a su degradación (Zhang *et al.* 2007), como por su insolubilidad en la mayoría de los disolventes (Zakzeski *et al.* 2010). Actualmente, los principales procesos de biorrefinería de materiales lignocelulósicos son la producción de celulosa y la producción de bioetanol, aunque los residuos de lignina de estos procesos (contenidas en las lejías negras y residuos forestales) también son susceptibles de uso. A continuación se describen más en detalle la obtención de pasta de celulosa para la producción de papel, y la obtención de bioetanol.

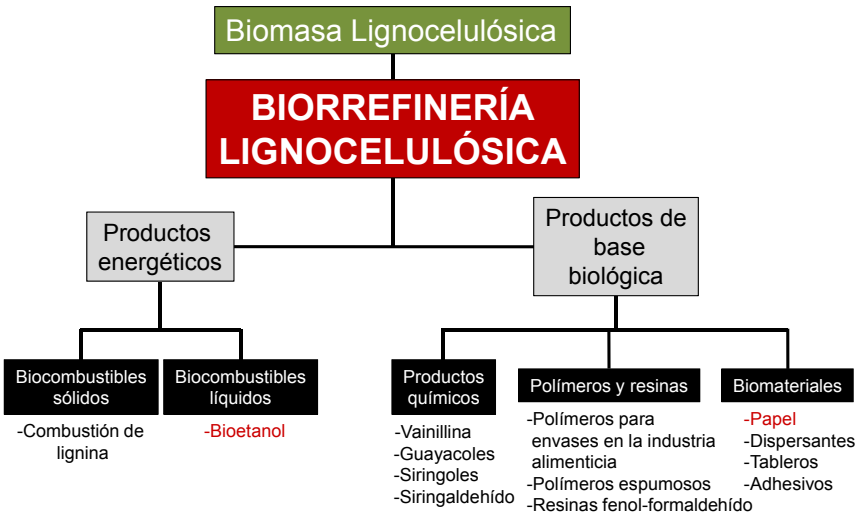


Figura 15. Esquema de una biorrefinería lignocelulósica. En negro ejemplos de productos obtenidos a partir de la lignina y en rojo ejemplos de productos obtenidos a partir de la celulosa.

I.2.1. Producción de pasta de papel

El principal uso no alimenticio de la biomasa vegetal es la producción de pasta de celulosa a partir de la cual es posible obtener una variada gama de productos como papel ondulado para embalaje y envases, papel para escribir e imprimir, cartón para embalaje, papel prensa, etc. La fabricación de la pasta de papel consiste básicamente en la separación de las fibras de celulosa, que se encuentran cementadas por la lignina. Existen muchos procedimientos que se han ido desarrollando y mejorando a lo largo del tiempo, que presentan ventajas e inconvenientes que han de ser evaluados conforme al tipo de producto final que se desea obtener, teniendo en cuenta parámetros tales como resistencia mecánica del papel a la rotura, rasgado, rozamiento, plegado, rugosidad, blancura, deteriorabilidad, además del costo unitario del proceso, impacto medioambiental de la producción o el tipo de materia prima disponible. La obtención de productos celulósicos comprende fundamentalmente el proceso de pasteado y el proceso de blanqueo.

Pasteado

El proceso de pasteado destruye o debilita los enlaces interfibras en el vegetal para la separación de las fibras de celulosa mediante procesos mecánicos y/o químicos o la combinación de ambos (**Tabla 1**).

Pasteado mecánico

El pasteado mecánico separa las fibras por fragmentación mecánica, utilizando molinos y refinadores de discos, lo que supone un considerable gasto energético. Prácticamente todos los constituyentes estructurales originales del vegetal, celulosa y hemicelulosas (deseados, blancos e hidrofílicos) y lignina (indeseable, oscura e hidrofóbica), permanecen en la pasta, por lo que el rendimiento obtenido es próximo al 95% (García Hortal 2007).

Tabla 1. Clasificación de los procesos de obtención de pastas (adaptado de García Hortal 2007).

Nombre común	Propiedades pastas	Usos	Rendimiento %	Requerimiento energía kWh/t
Mecánicos - De muela - Mecánica de refinos - Termomecánica	Fibras lignificadas, rígidas y poca capacidad enlace Alta opacidad y suavidad Buena imprimabilidad Volumen específico Baja resistencia	Mezcla con pastas químicas Papel prensa, revistas, libros Tejidos absorbentes (tisúes) Cartones para cajas, aislantes y para construcción	91-95	1800
Semiquímicos - Sulfito neutro - Kraft - Sin azufre - Sosa	Buena rigidez, dureza y moldeabilidad Baja capacidad absorción y tacto áspero	Papel para ondular, tubos y cartones para cajas	65-85	200
Químicos - Kraft - Polisulfuro - Sosa-AQ - Sosa-oxígeno - Sulfito ácido - Bisulfito - Sulfito múltiples estadios	**Kraft** Alta resistencia Pastas crudas oscuras **Sufito ácido/Bisulfito** Fácil blanqueo Refinado más fácil. Menos resistentes que kraft pero mayor rendimiento	**Kraft** Papeles para sacos, embalajes. Pastas blanqueadas **Sulfito ácido** Pastas alta calidad. Pastas para disolver **Bisulfito** Pastas calidad media, tisúes. Impresión-escritura, cristal. Reforzante en papel prensa	40-60	100

Pasteado químico

El pasteado químico utiliza reactivos químicos a altas temperaturas y presiones degradando y disolviendo gran parte de la lignina. El material conserva la mayor parte de su celulosa y algunas hemicelulosas. El rendimiento en el pasteado químico es del 40-60%, sin embargo, la pasta se blanquea mejor y el producto es más resistente y de mejor calidad que en el caso de los procesos mecánicos (Sjöström 1993). Existen varios tipos de pasteado químico:

- Proceso a la sosa
  El proceso a la sosa es el más antiguo y el más simple de los procesos químicos alcalinos. En este proceso, la fibra se somete a un proceso de cocción con sosa cáustica y vapor a alta presión y temperatura. La sosa (NaOH) es un producto muy útil para la deslignificación de materias primas vegetales. También se puede utilizar antraquinona (AQ) como catalizador ya que acelera el proceso de deslignificación y estabiliza los carbohidratos, mejorando los rendimientos respecto al proceso en ausencia de antraquinona (Abarca y Blanco 2008).

- Proceso kraft
  El proceso kraft para la obtención de pasta de papel es un proceso químico alcalino que deriva del proceso a la sosa. En primer lugar se cargan en el digestor las astillas con el denominado licor blanco (mezcla de NaOH y NaS_2), y se calienta hasta llegar a temperaturas de entre 160 y 180 ºC, manteniéndose estas condiciones en función del tipo de madera, el grado de deslignificación y el rendimiento de pasta requerido. Una vez terminada la cocción, la mezcla de pasta y astillas no digeridas se separan, y la pasta pasa a una etapa de lavado. El licor resultante de la cocción, denominado licor negro, se somete al ciclo de regeneración. En el proceso kraft se elimina mucha cantidad de lignina por lo que el rendimiento obtenido es bajo (40-60%), pero la resistencia de la pasta es muy alta. Para este proceso se pueden utilizar todo tipo de maderas, aunque los mejores resultados se obtienen con maderas de frondosas.

- Proceso al sulfito
  El proceso al sulfito emplea los reactivos H_2SO_3 o $Ca(HSO_3)_2$, producidos a partir de SO_2 y $CaCO_3$. Este proceso dominó la industria papelera desde finales del siglo XIX hasta casi mediados del XX, pero actualmente representa sólo una pequeña proporción de la producción global de pasta de papel.

- Proceso organosolv
  El proceso organosolv utiliza solventes orgánicos para la deslignificación. Se han empleado multitud de disolventes orgánicos (etanol, metanol, butanol, alcohol bencílico, glicerol, glicol, fenol,

acetona, dioxano, dimetilsulfóxido…) puros o en disolución acuosa, con la adición o no de catalizadores. Estos procesos presentan una mayor selectividad y por lo tanto, dan lugar a rendimientos mayores. También permiten la utilización de cualquier materia prima fibrosa (coníferas, frondosas y plantas no madereras). Sin embargo, las propiedades de resistencia de las pastas organosolv son inferiores a las pastas kraft. Los elevados precios de los reactivos, la dificultad en su recuperación y en muchos casos su elevada toxicidad, ha favorecido el uso de alcoholes alifáticos de bajo peso molecular (etanol y metanol) como solventes para los procesos organosolv (Pan *et al.* 2005).

Pasteado semiquímico

El pasteado semiquímico utiliza una combinación de métodos químicos y mecánicos. La madera está parcialmente cocida con productos químicos y el resto del pasteado se lleva a cabo mecánicamente.

Blanqueo

La mayoría de las pastas de celulosa una vez obtenidas son demasiados oscuras para ser utilizadas en productos que requieren un nivel de blancura elevado (por ejemplo calidades de impresión o tisúes). En el proceso de blanqueo se trata químicamente la pasta de celulosa, cuyo objetivo es eliminar o modificar (decolorar) las sustancias responsables del color, básicamente la lignina residual (restos de lignina que permanecen en la pasta). Se realiza en varias etapas según el punto de blancura que se desee (**Figura 16**).

Figura 16. Diferentes grados de blancura de una pasta de celulosa

En el blanqueo de las **pastas químicas**, la lignina residual (del orden del 2-5%) se puede modificar o eliminar casi completamente mediante un tratamiento en varias etapas sucesivas. Los reactivos comerciales más utilizados para el blanqueo de las pastas químicas son el cloro, el hipoclorito de sodio y el dióxido de cloro. Sin embargo, el desarrollo de leyes ambientales más restrictivas con respecto a los procesos contaminantes (Brooks *et al*. 1994) y en especial contra los compuestos clorados, catalogados como contaminantes prioritarios por la agencia de protección del medioambiente (EPA), por su persistencia en el suelo y el agua, ha llevado a la industria papelera a introducir una serie de modificaciones en sus plantas de blanqueo dando lugar primero a la aparición de las secuencias de blanqueo libres de cloro elemental (ECF) y posteriormente a las secuencias totalmente libres de cloro (TCF). Las secuencias de blanqueo TCF utilizan como agentes blanqueantes oxígeno, ozono o peróxido de hidrógeno.

Los métodos de blanqueo de las **pastas mecánicas**, que tienen porcentajes muy elevados de lignina (prácticamente toda la lignina original, entre 10-25%), difieren de los utilizados en el blanqueo de las pastas químicas. Este blanqueo se lleva a cabo mediante tratamientos que destruyen de manera selectiva los grupos cromóforos responsables del color de las pastas, pero sin eliminación significativa de la lignina, puesto que ocasionaría una pérdida importante de rendimiento y un consumo muy importante de productos químicos costosos. Los agentes de blanqueo más utilizados son el peróxido de hidrógeno (oxidante) y los hidrosulfitos (reductores) (García Hortal 2007). Las pastas de alto rendimiento blanqueadas conservan prácticamente toda la lignina original pero en un estado que no proporciona color a las pastas. Mediante exposición a radiaciones ultravioletas (luz solar) se regeneran los grupos cromóforos y las pastas amarillean de nuevo (reversión de blancura).

I.2.2. Producción de biocombustibles de segunda generación

El modelo energético actual (**Figura 17**) está basado en un 80% en energías de origen fósil como el petróleo, el carbón y el gas natural. Los combustibles fósiles se caracterizan por ser fuentes no renovables de energía y por su impacto negativo sobre el medio ambiente, por ello deben desarrollarse formas de energía alternativas para hacer frente al agotamiento de energía fósil y respetar el medio ambiente.

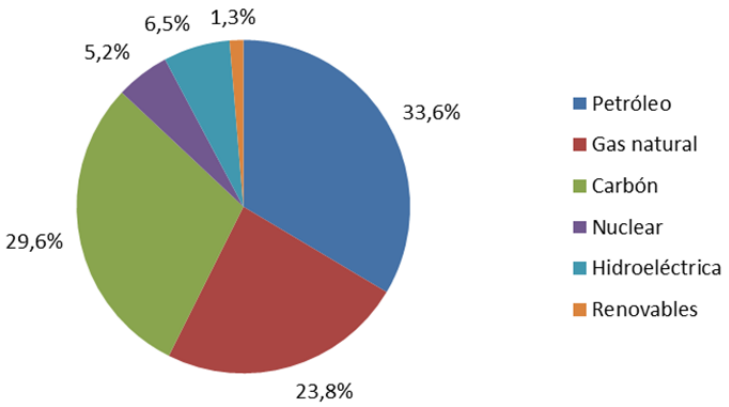


Figura 17. Consumo mundial de energía primaria por fuentes. Gráfico elaborado a partir de los datos del 2010 que aparecen en el documento BP Statistical Review of World Energy, de Junio de 2011.

Con este panorama, no es de extrañar que los biocombustibles se presenten como la alternativa más prometedora debido a sus ventajas (**Figura 18**) con respecto a los combustibles fósiles. Los biocombustibles más importantes son:

Bioetanol (bioalcohol): Formado a partir de la fermentación de los azúcares que se encuentran en determinados cultivos vegetales (caña de azúcar, remolacha, etc.). Se puede emplear como biocombustible de diversas maneras, aunque quizá su aplicación más conocida sean los biocombustibles EXX. Estos biocombustibles se obtienen mezclando un porcentaje de bioetanol con otro de gasolina convencional. Así, el E5 es una mezcla de 5% de bioetanol y 95% de gasolina normal, el E10 es una mezcla de 10% de bioetanol y 90% de gasolina normal, y así sucesivamente.

Biodiésel (bioaceite): Se obtiene a partir de aceites vegetales mediante procesos de esterificación y transesterificación (principalmente aceite de colza y soja), y mezclado con diésel normal genera unos

biocombustibles que pueden ser utilizados en los motores de este tipo sin ninguna modificación. Los rendimientos son muy similares a los del diésel convencional, pero con una reducción sustancial de las emisiones contaminantes.

Figura 18. Ventajas de los biocombustibles.

Los biocombustibles también presentan algunos inconvenientes, debido principalmente a que se trata de una industria que aún no está plenamente desarrollada. Aunque si bien son reconocidas sus potencialidades, presentan una serie de amenazas éticas concentradas específicamente en los llamados **"biocombustibles de primera generación"** que utilizan como materia prima cultivos básicos en la alimentación, entre los que destacan el maíz, caña de azúcar o la soja, lo que conlleva al aumento de precios de dichos alimentos.

Tratando de encontrar alternativas a estos cultivos alimenticios se están desarrollando los llamados **"biocombustibles de segunda generación"** que usan materias primas que no son fuentes de alimentación. Al mismo tiempo, los biocombustibles de segunda generación tienen un alto poder de reducción de emisiones respecto a los combustibles fósiles que sustituyen.

Entre ellos destaca el bioetanol de segunda generación o alcohol lignocelulósico, que se obtiene a partir de la celulosa de las plantas (residuos agrícolas, maderas, cultivos energéticos, etc.) por sacarificación y posterior fermentación de los azúcares obtenidos (**Figura 19**). Sin embargo, la lignina de la pared celular supone una barrera para la hidrólisis de la celulosa. Por ello, se requiere separar la lignina de la celulosa para lo que se pueden emplear diversos pretratamientos (**Figura**

19). Los pasos para obtener el bioetanol de segunda generación son: pretratamiento, hidrólisis y fermentación.

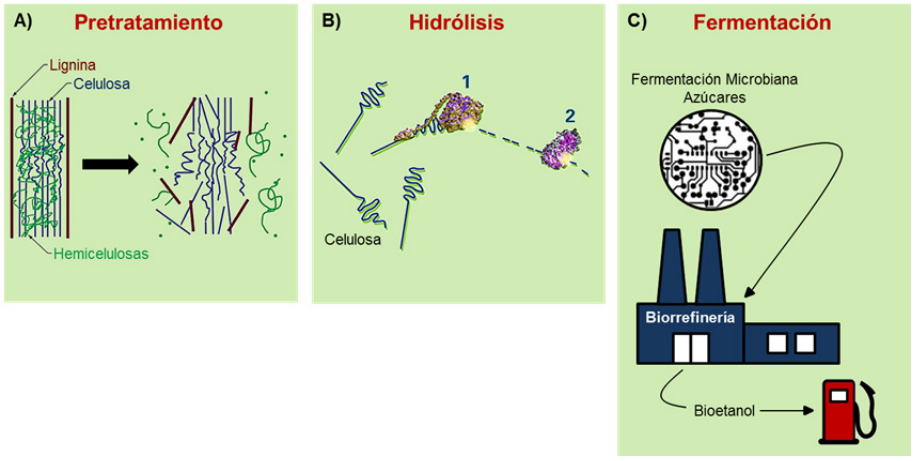


Figura 19. Distintos pasos para la producción de bioetanol a partir de material lignocelulósico: (A) pretratamiento, (B) hidrólisis de la celulosa con celulasas (1) produciendo celobiosa que libera a glucosa por la acción de β-glucosidasas (2), y (C) producción de bioetanol por fermentación de la glucosa.

Pretratamientos

Cuando se parte de un material lignocelulósico, el pretratamiento es un paso esencial para la obtención de azúcares fermentables en la etapa de hidrólisis. El objetivo del tratamiento previo es separar la lignina de la celulosa y hemicelulosas, para hacer a los carbohidratos más accesibles a las enzimas hidrolíticas. Los requisitos de un pretratamiento idóneo para la obtención de bioetanol fueron recogidos por Carvalheiro *et al.* (2008) y Yang y Wyman (2008):

- Aumentar la porosidad y el área específica de la biomasa para facilitar la accesibilidad de los reactivos o las enzimas que se emplean durante la hidrólisis.
- Reducir el pretratamiento mecánico (reducir gastos energéticos).
- Generar celulosa activa (reducir la cristalinidad de la celulosa, apta para los posteriores procesos de hidrólisis y fermentación).
- Desacoplar las hemicelulosas y maximizar el rendimiento de pentosas en forma no degradada.

- Obtener hidrolizados con un bajo nivel de inhibidores, y minimizar las pérdidas de nutrientes necesarios para la fermentación.
- Emplear reactores con una alta carga de sólidos por volumen.
- Minimizar los vertidos y contaminantes residuales.

Entre los distintos pretratamientos existentes, se puede distinguir los pretratamientos físicos, químicos, físico-químicos, así como los pretratamientos biológicos que se describen a continuación.

Pretratamientos físicos

Los pretratamientos físicos, tales como la trituración mecánica, la pulverización por martilleo y la extrusión, buscan romper la estructura lignocelulósica y reducir el tamaño de partícula y cristalinidad de la celulosa con el fin de aumentar la superficie específica y reducir el grado de polimerización. En general, los pretratamientos físicos son efectivos, pero son largos y/o costosos. La energía necesaria para la reducción del tamaño de las partículas puede alcanzar hasta una tercera parte de la energía total necesaria para la producción de bioetanol (Aden *et al.* 2002; US Department of Energy 1993).

Pretratamientos químicos

- Pretratamientos alcalinos
  Estos pretratamientos aumentan la digestibilidad de la celulosa y son más eficaces que los procesos ácidos o hidrotermales para la solubilización de la lignina (Carvalheiro *et al.* 2008). El pretratamiento alcalino puede llevarse a cabo a temperatura ambiente y tiempos que van de segundos a días. Causan una menor degradación del azúcar que el pretratamiento ácido y ha demostrado ser más eficaz en residuos agrícolas que en los materiales de madera (Kumar *et al.* 2009). Sin embargo, la posible pérdida de azúcares fermentables y la producción de compuestos inhibidores debe ser tomada en consideración para optimizar las condiciones de pretratamiento. Hidróxido de sodio, hidróxido de potasio, hidróxido de calcio e hidróxido de amonio son pretratamientos alcalinos adecuados. La adición de un agente oxidante (oxígeno/H_2O_2) para el tratamiento previo alcalino (NaOH/$Ca(OH)_2$) puede mejorar el rendimiento al favorecer la eliminación de la lignina (Carvalheiro *et al.* 2008).

- Pretratamientos ácidos
  El objetivo principal de los pretratamientos ácidos es solubilizar la fracción de hemicelulosa para hacer que la celulosa sea más accesible a las enzimas. Este tipo de tratamientos previos se pueden realizar con ácido concentrado o diluido pero la utilización de ácido concentrado es menos atractivo debido a la formación de compuestos

inhibidores y la corrosión de reactores y equipos. Los altos costos de operación y mantenimiento reducen el interés de la aplicación del pretratamiento con ácido concentrado a escala comercial (Wyman 1996). El tratamiento previo con ácido diluido aparece como método más favorable para aplicaciones industriales. Se han obtenido rendimientos altos con ácido sulfúrico, que es el ácido más estudiado. Los ácidos clorhídrico, fosfórico y nítrico también se han probado (Mosier *et al.* 2005a). Ácidos orgánicos tales como ácido fumárico o maleico están apareciendo como alternativas para mejorar la hidrólisis de la celulosa.

- Ozonólisis

  El ozono es un potente oxidante que muestra una alta eficacia en la deslignificación (Sun y Cheng 2002). Este pretratamiento no conduce a la formación de compuestos inhibidores que puedan afectar a la posterior hidrólisis y fermentación. Un inconveniente importante a considerar es la gran cantidad de ozono necesario, que puede hacer que el proceso sea económicamente inviable (Sun y Cheng 2002).

- Organosolv

  Consiste en la utilización de mezclas de disolventes orgánicos o acuosos, tales como metanol, etanol, acetona, glicol de etileno y alcohol tetrahidrofurfurílico, con el fin de solubilizar la lignina y proporcionar celulosa adecuada para la hidrólisis enzimática (Zhao *et al.* 2009). La principal ventaja del proceso organosolv es la recuperación de la lignina relativamente pura como un subproducto (Zhao *et al.* 2009). Los solventes deben ser separados, ya que podrían tener efectos inhibidores en la hidrólisis enzimática y los microorganismos fermentativos (Sun y Cheng 2002). El alto precio de los disolventes es otro factor importante a considerar para aplicaciones industriales.

- Líquidos iónicos

  Los líquidos iónicos son sales con una temperatura de fusión por debajo del punto de ebullición del agua. Típicamente están compuestos de cationes orgánicos y aniones inorgánicos. Los líquidos iónicos son conocidos como disolventes “verdes” dado que no forman gases tóxicos o explosivos. Los hidratos de carbono y la lignina se pueden disolver de forma simultánea en los líquidos iónicos, como resultado, las interacciones no covalentes entre los polímeros de celulosa, hemicelulosas y lignina se interrumpe eficazmente y reduce al mínimo la formación de productos de degradación. Para la aplicación a gran escala de los líquidos iónicos, el desarrollo de métodos de reciclado es un requisito indispensable. Las técnicas deben ser desarrolladas para recuperar hemicelulosas y lignina de las soluciones después de la extracción de la celulosa (Hayes 2009).

Pretratamientos físico-químicos

- Explosión con vapor

  Es el pretratamiento físico-químico más ampliamente empleado. La explosión con vapor consiste básicamente en calentar el material lignocelulósico en un reactor con vapor a alta presión (20-50 bares, 210-290ºC) durante unos minutos. Después, la bajada brusca de la presión hace expandir el vapor dentro de la biomasa para facilitar la impregnación de agua en la matriz lignocelulósica. Los factores más importantes que afectan a la explosión con vapor son el tamaño de partícula, temperatura, tiempo de residencia y el efecto combinado de la temperatura y el tiempo. Cabe destacar como características más atractivas el menor impacto ambiental, menor inversión de capital, mayor potencial de eficiencia energética, productos químicos y condiciones del proceso menos peligrosas y la recuperación completa de los carbohidratos (Avellar y Glasser 1998). Aunque la posibilidad de evitar catalizadores ácidos se considera una ventaja, la adición de un catalizador ácido ha sido también descrito como una manera de aumentar la digestibilidad de la celulosa, para mejorar la hidrólisis de la hemicelulosa y, dependiendo de la temperatura, para disminuir la producción de compuestos de degradación (Sun y Cheng 2002). Los principales inconvenientes de la explosión con vapor son la degradación parcial de la hemicelulosa y la generación de algunos compuestos tóxicos que pudieran afectar a las siguientes etapas de hidrólisis y fermentación (Oliva *et al.* 2003).

- Agua líquida caliente

  Se basa en aplicar presión para mantener el agua en estado líquido a temperaturas elevadas (160-240ºC) y provocar alteraciones en la estructura lignocelulósica. El objetivo principal es solubilizar la hemicelulosa para hacer que la celulosa sea más accesible y para evitar la formación de inhibidores. En general, este pretratamiento es atractivo debido a que no requiere la presencia de catalizador, construcción de reactores de bajo costo a causa del bajo potencial de corrosión y baja concentración de hemicelulosa solubilizada y productos de lignina. Sin embargo, el agua exigida en el proceso y los altos requisitos energéticos hacen que no se desarrolle a escala comercial.

- Explosión amoniacal de la fibra (AFEX)

  En el proceso AFEX se emplea amoníaco a temperaturas entre 60-100ºC, y alta presión durante un período de tiempo variable. Se libera la presión, lo que resulta en una rápida expansión del amoníaco que causa inflamación y ruptura física de las fibras de la biomasa y descristalización parcial de la celulosa. Recuperar y reciclar el amoníaco es factible a pesar de su alta volatilidad (Teymouri *et al.* 2005), pero la complejidad asociada y los costes de recuperación del

amoníaco pueden ser significativos en relación con el potencial comercial del pretratamiento AFEX (Eggeman y Elander 2005; Mosier *et al.* 2005b). Una de las principales ventajas es que no hay formación de inhibidores. En condiciones óptimas el proceso AFEX puede conseguir una conversión de más del 90% de celulosa y hemicelulosas en azúcares fermentables. Otro tipo de proceso que utiliza el amoníaco es conocido como percolación reciclada de amoníaco en el que el amoníaco acuoso (5-15% en peso) pasa a través de un reactor relleno con biomasa. Un reto importante para este proceso es disminuir la carga de líquido o la temperatura del proceso para reducir el costo de la energía.

- Oxidación húmeda

  La oxidación húmeda es un pretratamiento oxidativo que emplea oxígeno o aire como catalizador. La oxidación se lleva a cabo durante 10-15 minutos a temperaturas desde 170 hasta 200ºC y a presiones de 10 a 12 bares de O_2 (Olsson *et al.* 2005). En general, se logra una formación de inhibidores baja y una eliminación eficaz de la lignina.

- Pretratamiento con microondas

  Este pretratamiento se lleva a cabo por la inmersión de la biomasa en reactivos químicos diluidos y exponiendo la suspensión a la radiación de microondas por tiempos de residencia que varían de 5 a 20 minutos (Keshwani 2009).

- Pretratamiento con ultrasonido

  El efecto de los ultrasonidos sobre la biomasa lignocelulósica se ha empleado para la extracción de celulosa, hemicelulosas y lignina. Algunos investigadores han demostrado que la sacarificación de la celulosa se ha mejorado de manera eficiente por el pretratamiento ultrasónico (Yachmenev *et al.* 2009).

- Explosión con CO_2

  Su metodología es igual a la del pretratamiento de explosión con vapor, pero con la adición de dióxido de carbono (CO_2). El CO_2 formaría ácido carbónico aumentando la velocidad de hidrólisis. En la práctica, este pretratamiento no ha ofrecido mejores rendimientos que otros similares; sin embargo, tiene la ventaja de que no forma compuestos inhibitorios como en el caso de la explosión con vapor (Sun y Cheng 2002).

Pretratamientos biológicos

Además de los pretratamientos físicos, químicos y físico-químicos, existen pretratamientos biológicos que usan hongos y enzimas. Estos pretratamientos se describen con más detalle en el apartado I.3.

Hidrólisis

La hidrólisis o sacarificación consiste en la rotura de los carbohidratos, presentes en los materiales lignocelulósicos (celulosa y hemicelulosas), en sus azúcares individuales (glucosa, xilosa, arabinosa, galactosa, etc.). La hidrólisis se puede llevar a cabo química o enzimáticamente.

En la hidrólisis ácida, el ácido sulfúrico y el ácido clorhídrico son ácidos potenciales para la hidrólisis de materiales lignocelulósicos (Aguilar *et al.* 2002). Sin embargo, el uso de estos ácidos concentrados conlleva un determinado nivel de corrosión en los reactores y además son tóxicos, corrosivos y peligrosos (Velmurugan y Muthukumar 2011). Por este motivo, se han desarrollado procesos de hidrólisis enzimática, mediante el uso de celulasas que atacan a las fibras de celulosa de los tejidos vegetales (Bhat 2000). Estas enzimas son producidas por hongos como *Trichoderma reesei* y *Aspergillus niger* y/o bacterias, tales como *Clostridium cellulovorans* (Arai *et al.* 2006). La mayoría de investigaciones para la producción de celulasas comerciales se han centrado en los hongos ya que la mayoría de las bacterias relevantes son anaerobias con una baja tasa de crecimiento. Debido a las características de la celulosa no es posible obtener glucosa a partir de celulosa por la acción directa de un solo tipo de celulasas (Wyman 1996). Para ello es necesario la acción de diferentes tipos de celulasas (endo-glucanasas, exo-glucanasas y β-glucosidasas) que actúan sinérgicamente sobre la celulosa (Wright 1988). La endo-glucanasa y la exo-glucanasa rompen los fragmentos de celulosa por el interior y los extremos, respectivamente, en moléculas de celobiosa que a continuación se dividen por la acción de la β-glucosidasa en glucosas individuales. El pretratamiento de materiales celulósicos y las condiciones en la que se realiza la hidrólisis, como temperatura y pH, se encuentran entre los factores que más influyen en la eficiencia de la hidrólisis enzimática. A menudo en los estudios de laboratorio se utilizan dosis de celulasas de 10 FPU/g y de β-glucosidasa de 500 nkat/g, ya que resulta una hidrólisis eficaz con un alto rendimiento de glucosa en un tiempo (48-72 horas) y coste de enzima razonable.

Fermentación

La conversión a bioetanol se lleva a cabo por fermentación de los monosacáridos (pentosas y hexosas) liberados por la hidrólisis. La fermentación se produce mediante el uso de microorganismos apropiados. El microorganismo ideal debe tener una amplia aplicación sobre diferentes sustratos, alto rendimiento y productividad de etanol, tolerancia a los inhibidores presentes en los hidrolizados y a altas concentraciones de etanol, y capacidad para fermentar azúcares a altas temperaturas (Hahn-Hägerdal *et al.* 2007). Los microorganismos más

usados para la producción de etanol son la levadura *Saccharomyces cerevisiae* y la bacteria *Zymomona mobilis* (Claassen *et al.* 1999) que ofrecen altos rendimientos de etanol (90-97%) y alta tolerancia al etanol.

El proceso de hidrólisis enzimática y la fermentación se puede realizar utilizando diferentes estrategias: hidrólisis y fermentación separada (SHF), hidrólisis y fermentación simultáneas (SSF) y conversión microbiana directa (DMC). En SHF, la hidrólisis y la fermentación se llevan a cabo en recipientes separados bajo sus propias condiciones óptimas, sin embargo, con este proceso está asociado la inhibición de la actividad enzimática por parte del producto final y los problemas de contaminación. Con el fin de eliminar los inconvenientes del proceso de SHF, se ha desarrollado el proceso SSF, que combina la hidrólisis y la fermentación en un mismo recipiente. Los azúcares producidos durante la hidrólisis se fermentan inmediatamente a etanol y por lo tanto, se pueden evitar los problemas asociados con la acumulación de azúcar, la inhibición de la enzima, así como la contaminación (Galbe y Zacchi 2002; Ohgren *et al.* 2007). Otra ventaja de la SSF sobre SHF es la reducción de costes debido al uso de un solo reactor. El principal inconveniente de SSF son las diferentes temperaturas óptimas de los procesos de hidrólisis y fermentación. La mayoría de las levaduras de fermentación tienen una temperatura óptima alrededor de 30-35°C, mientras que las enzimas que hidrolizan muestran actividades óptimas alrededor de 50°C (Kadar *et al.* 2004). En DMC no se añade la enzima de manera externa, sino que el proceso de hidrólisis está mediado por las enzimas producidas por los microorganismos (Demirbas 2005). Los azúcares generados son convertidos inmediatamente en etanol. El proceso de conversión microbiana directa tiene la desventaja de un bajo rendimiento en etanol.

I.2.3. Problemas que limitan el aprovechamiento industrial de los materiales lignocelulósicos

Algunos de los componentes presentes en los materiales lignocelulósicos como la lignina y los lípidos pueden limitar su aprovechamiento industrial.

Problemática de la lignina

Como se ha mencionado anteriormente, la lignina forma un conglomerado con los carbohidratos formando una barrera que evita el acceso a la celulosa. En la producción de biocombustibles a partir de lignocelulosa, la presencia de lignina supone un problema, ya que obstaculiza el acceso de las enzimas hidrolíticas (celulasas y β-glucosidasa) a los carbohidratos.

Por otro lado, la lignina es responsable del color oscuro de las pastas químicas de celulosa, un problema que se ha agravado debido a la utilización de reactivos de blanqueo menos agresivos.

Problemática de los lípidos

En la industria papelera existe otro problema relacionado con los extraíbles lipofílicos presentes en los materiales lignocelulósicos. Estos compuestos forman unos depósitos comúnmente denominados depósitos de *pitch*, que afectan negativamente al proceso de fabricación de pasta de celulosa dando lugar a importantes pérdidas económicas por depositarse en las máquinas de papel (que obligan a realizar paradas en la producción) y en la misma pasta (que da lugar a pastas contaminadas) (Allen 1980; Hillis y Sumimoto 1989; Back y Allen 2000), reduciendo drásticamente la calidad del producto final (**Figura 20**).

Además, algunos compuestos lipofílicos también tienen un impacto negativo sobre el medio ambiente, considerándose tóxicos cuando se liberan en los vertidos (Ali y Sreekrishnan 2001; Rigol *et al.* 2004). Estos problemas se han agravado debido a la utilización de reactivos de blanqueo menos agresivos. Cuando anteriormente se utilizaban reactivos clorados, muchos de estos compuestos lipofílicos se degradaban o estaban presentes en concentraciones más bajas.

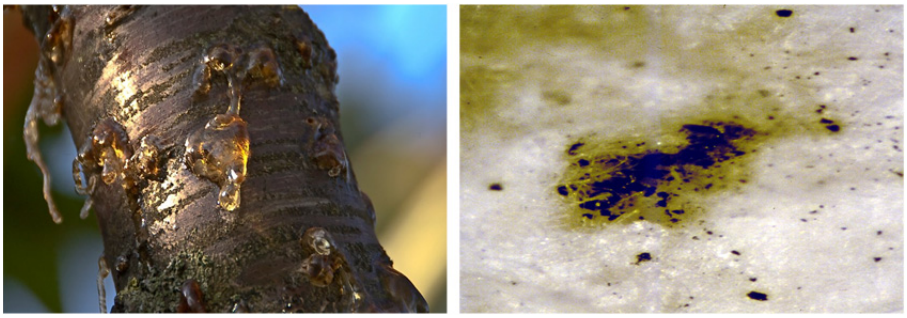


Figura 20. Imagen de resina en un árbol (izquierda) y de un depósito de *pitch* en una pasta kraft TCF (derecha).

I.3. BIOTECNOLOGÍA EN LA BIORREFINERÍA DE LA LIGNOCELULOSA

La eliminación de la lignina y los lípidos es una cuestión técnica importante en la producción de celulosa en la industria papelera y la deslignificación un desafío clave para la conversión de material lignocelulósico en combustibles líquidos como el bioetanol de segunda generación. En la actualidad, se elimina la lignina y los lípidos mediante el uso de tratamientos físicos, químicos o físico-químicos caracterizados por su alto consumo energético y su impacto negativo sobre el medio ambiente. A diferencia de ellos, los procesos biotecnológicos, como los tratamientos con hongos y con enzimas, ofrecen la posibilidad de usar tecnologías respetuosas con el medio ambiente y de gran interés desde un punto de vista industrial. Aunque muchas de las aplicaciones enzimáticas están aún en estado de investigación y desarrollo, algunas ya han comenzado a aplicarse en la industria.

I.3.1. Biodegradación de lignina y lípidos con hongos

Entre los hongos capaces de degradar la lignina están algunos hongos ascomicetos, como *Chrysonilia sitophyla* (Rodríguez *et al.* 1997), y deuteromicetos, como *Fusarium proliferatum* (Regalado *et al.* 1997). Estos hongos producen un tipo de degradación denominada podredumbre blanda que, generalmente, tiene lugar en angiospermas con un alto porcentaje de humedad y sobre materiales que ya han comenzado a ser degradados por otros microorganismos (Blanchette 1995; Daniel 2003). Sin embargo, los basidiomicetos de podredumbre blanca constituyen el grupo más hábil de microorganismos a la hora de llevar a cabo una eliminación rápida y efectiva de la lignina con una mínima pérdida de celulosa (Kirk y Farrell 1987). El término podredumbre blanca hace referencia al aspecto que presenta la madera tras su colonización: la eliminación de la lignina hace que adquiera una tonalidad más blanquecina y un aspecto fibroso debido a la exposición de la celulosa (Martínez *et al.* 2011). Estos hongos de podredumbre blanca, durante su crecimiento en la naturaleza utilizan dos sistemas enzimáticos extracelulares para degradar la biomasa lignocelulósica: un sistema hidrolítico (produce hidrolasas) que degradan los polisacáridos, y un sistema ligninolítico oxidativo, que degrada la lignina (Sánchez 2009). En la mayoría de los casos, la eliminación de lignina por hongos de podredumbre blanca es paralela a la degradación de polisacáridos, en el llamado patrón de degradación simultánea (Otjen y Blanchette 1986). Sin embargo, unas pocas especies de hongos de podredumbre blanca son capaces de eliminar la lignina sin causar una pérdida significativa de polisacáridos, en un patrón llamado de degradación selectiva, de mayor interés biotecnológico. Por otro lado, los hongos de podredumbre parda han desarrollado una estrategia de degradación diferente al ser capaces

de degradar la mayor parte de la celulosa y hemicelulosas, dejando el polímero de lignina intacto. La madera se enriquece en lignina y presenta un color desde marrón rojizo oscuro a dorado, y de ahí su nombre (Martínez *et al.* 2011).

El pretratamiento con hongos de podredumbre blanca para la eliminación de la lignina durante la producción de pasta de papel supone una alternativa al tratamiento químico del material lignocelulósico, dando lugar a un tipo de pasta de papel denominada como biomecánica (Kirk *et al.* 1992). Este pretratamiento supone una serie de ventajas energéticas, físicoquímicas y medioambientales en la producción de este tipo de pasta (Akhtar *et al.* 1998). El tiempo de tratamiento debe de ser suficiente para reducir el contenido en lignina, sin ser excesivo, para evitar la pérdida de celulosa. Con la utilización de hongos ligninolíticos como *Ceriporiopsis subvermispora* se han conseguido ahorros de hasta un 40% de energía en el refino y una disminución de la toxicidad de los efluentes durante el tratamiento de maderas de coníferas y frondosas (Blanchette *et al.* 1990; Akhtar *et al.* 1993). Los hongos del género *Pleurotus* también se han estudiado para su posible utilización en el pretratamiento de paja de trigo como materia prima alternativa para la fabricación de pasta de papel (Martínez *et al.* 1994). En estos estudios se comprobó que algunas especies del género *Pleurotus* eran capaces de degradar la lignina sin alterar significativamente el contenido en polisacáridos de la paja de trigo. Su utilización, especialmente en el caso de *P. eryngii*, supondría un ahorro en reactivos químicos y energía empleados durante la fabricación de pasta semiquímica a partir de estos residuos (Camarero *et al.* 1998).

Por otro lado, los pretratamientos biológicos para la producción de biocombustibles de segunda generación emplean organismos, principalmente hongos de podredumbre blanca, parda y blanda que degradan la lignina y la hemicelulosa y muy poco la celulosa, más resistente que el resto de componentes (Sánchez 2009), siendo los hongos de podredumbre blanca los microorganismos más eficaces. El uso de *Phanerochaete chrysosporium*, uno de los hongos de podredumbre blanca mejor estudiados debido a su capacidad para degradar la lignina, tiene el inconveniente que requiere condiciones especiales de crecimiento para degradar la lignina (Kirk y Farrell 1987). Sin embargo, *P. eryngii* e *Irpex lacteus*, son hongos de podredumbre blanca capaces de crecer bajo condiciones menos restrictivas y degradar residuos lignocelulósicos de modo que puedan ser convertidos en etanol (Cohen *et al*. 2002; Salvachúa *et al*. 2013).

También, los problemas de *pitch* originados durante la producción de pasta de papel, y que han aumentado debido al uso de tecnologías más respetuosas con el medio ambiente y a la recirculación de las aguas de proceso, se han intentado solucionar mediante la utilización de microorganismos (Behrendt y Blanchette 1997; Farrell *et al.* 1993; Gao *et al.* 1994; Gutiérrez *et al.* 1999, 2001a). Entre estos microorganismos se

encuentra el Cartapip™ (**Figura 21B**), una cepa albina de *Ophiostoma piliferum*, un hongo ascomiceto que consigue reducir en un 40% el contenido de la fracción lipídica en madera de pino (Farrell *et al.* 1993). El Cartapip™ es efectivo en maderas de gimnospermas con alto contenido en triglicéridos, pero no lo es para la degradación de esteroles libres y esterificados, que son los lípidos mayoritarios en otras maderas, como el eucalipto (Gutiérrez *et al.* 1999). Otros hongos han sido también ensayados en el tratamiento de la madera por su capacidad para degradar esteroles (libres y esterificados) como *Phlebia radiata* (**Figura 21C**), *Bjerkandera adusta*, *Pleurotus pulmonarius* y *Ceriporiopsis subvermispora*. En maderas tratadas con estos hongos, se ha conseguido hasta un 75% de reducción de compuestos esteroidales en pastas kraft, y con una degradación mínima de la celulosa (Gutiérrez *et al*. 2000).

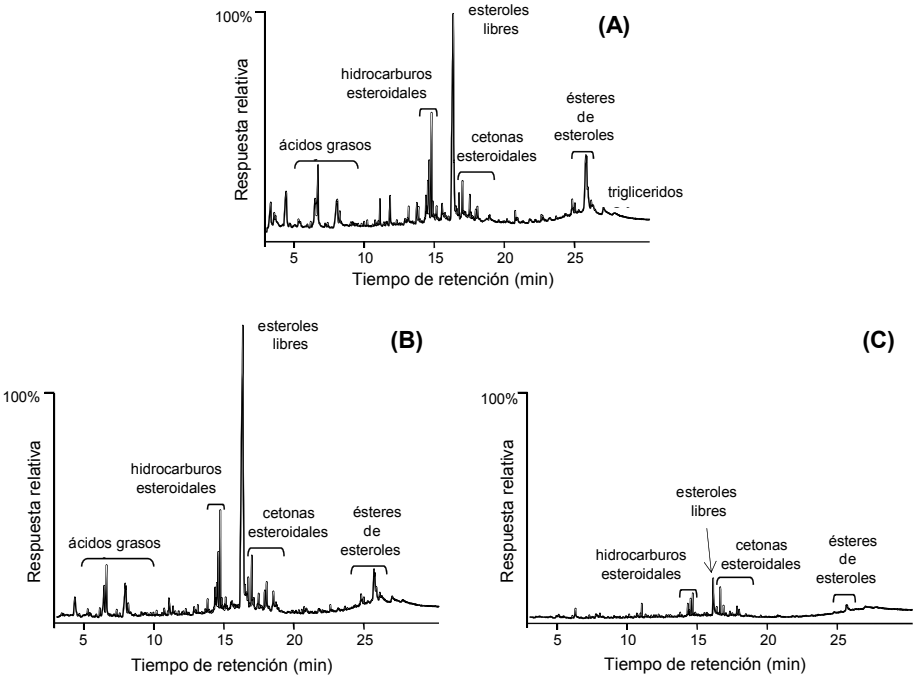


Figura 21. Análisis de cromatografía de gases de los extractos lipofílicos de la madera de eucalipto (*Eucalyptus globulus*) después del tratamiento con dos tipos de hongos: (A) control, (B) madera de eucalipto tratada con Cartapip^{TM} 97 (*O. piliferum*), y (C) madera de eucalipto tratada con *P. radiata*. Adaptado de Gutiérrez *et al.* (1999).

I.3.2. Biodegradación de lignina y lípidos con enzimas

La acción de las enzimas aisladas es más directa y selectiva que la de los hongos, ya que el tratamiento con hongos requiere tiempos de residencia más largos y puede conllevar una degradación más severa de los carbohidratos. Entre las enzimas más utilizadas para la eliminación de la lignina destacan las xilanasas, que se utilizan para limitar el uso de cloro en los procesos de blanqueo de la pasta (Viikari *et al.* 1994). Las xilanasas no actúan directamente sobre la lignina, sino catalizando la hidrólisis de los xilanos que se encuentran entre las microfibrillas de la celulosa y la lignina. Sin embargo, enzimas de los hongos de podredumbre blanca, de tipo oxidoreductasa (lacasas y peroxidasas) tienen mayor potencial que las xilanasas porque actúan directamente sobre la lignina. Las peroxidasas catalizan la oxidación de una gran variedad de compuestos tanto orgánicos como inorgánicos en presencia de peróxidos. Entre las peroxidasas ligninolíticas encontramos: la lignina peroxidasa (LiP), que oxida la lignina; la manganeso peroxidasa (MnP), que oxida compuestos de manganeso, abundantes en los materiales lignocelulósicos; y la peroxidasa versátil (VP), que se caracteriza por combinar propiedades catalíticas de las otras dos peroxidasas ligninolíticas (Ruiz-Dueñas *et al.* 1999).

También se han estudiado diversas enzimas para el control de los depósitos de *pitch* (Fischer y Messner 1992; Fischer *et al.* 1993; Fujita *et al.* 1992). Entre ellas se encuentran la Resinase™, una lipasa recombinante expresada en *Aspergillus oryzae* (Matsukura *et al.* 1990; Fujita *et al.* 1991), cuya aplicación, al igual que le ocurría al Cartapip™, es limitada ya que únicamente hidroliza los triglicéridos sin degradar ningún otro tipo de lípidos (como esteroles libres o esterificados). También se han descrito otras enzimas para la degradación de ésteres de esteroles, como la esterol esterasa aislada del hongo ascomiceto *Ophiostoma piceae*, que es capaz de hidrolizar simultáneamente tanto los esteres de esteroles como los triglicéridos en pastas (Calero-Rueda *et al*. 2004). Sin embargo, aunque las esterol esterasas hidrolizan los ésteres de esteroles, al mismo tiempo liberan esteroles libres que son tanto o más perjudiciales que los ésteres de esteroles en la formación de depósitos de *pitch.*

En la presente Tesis nos centramos en la utilización de las enzimas de tipo lacasas. Las lacasas (fenoloxidasas, EC 1.10.3.2) (**Figura 22**) constituyen un grupo de enzimas oxidativas, producidas por plantas y hongos, incluidos los basidiomicetos de podredumbre blanca responsables de la degradación de lignina en la naturaleza (Thurston 1994; Käärik 1965; Mayer y Staples 2002), aunque también se han descrito y caracterizado algunas lacasas bacterianas (Enguita *et al*. 2002). Las lacasas han sido objeto de interés tanto por su gran potencial, actuando directamente sobre la lignina y sobre un amplio rango de

compuestos lipofílicos, como por su uso de tecnologías más respetuosas con el medio ambiente.

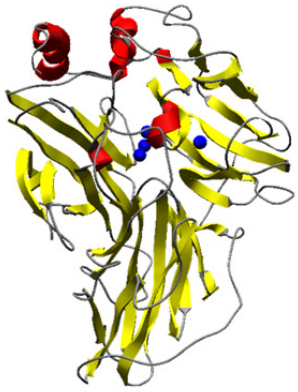
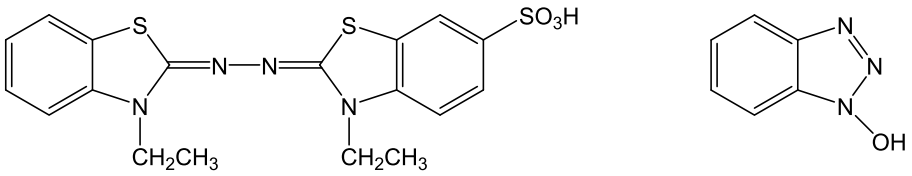


Figura 22. Representación esquemática de la estructura molecular de la lacasa de *Trametes versicolor* (entrada PDB 1GYC) mostrando los cobres catalíticos como esferas azules (Piontek *et al.* 2002), las regiones con estructura secundaria diferente corresponden a las hélices rojas y la lámina β se indica en amarillo.

La acción directa de las lacasas en principio está restringida a las unidades fenólicas, que constituyen únicamente un 20% del total de la lignina (Kawai *et al.* 1987a, 1987b). Sin embargo, el interés por estas enzimas se incrementó enormemente tras el descubrimiento del efecto de algunos compuestos sintéticos, tales como el ABTS (ácido 2,2′-azino-bis(3-etilbenzotiazolin-6-sulfónico)) (**Figura 23A**) (Bourbonnais y Paice 1990) y el HBT (1-hidroxibenzotriazol) (**Figura 23B**) (Call 1994), que amplían la acción de la lacasa a sustratos no fenólicos, lo que aumenta el potencial en la degradación de la lignina y de otros compuestos aromáticos (Call y Mücke 1997).



A **B**

Figura 23. Estructura de los mediadores sintéticos: (A) ABTS y (B) HBT.

La base del sistema lacasa-mediador es el uso de compuestos de bajo peso molecular que, una vez oxidados por la enzima a radicales estables, actúan como intermediarios redox oxidando compuestos que en principio no son sustratos de la lacasa (como las unidades no fenólicas de la lignina). El O_2 oxida a la lacasa (con formación de H_2O), después ésta se reduce oxidando al mediador hasta un radical libre estable que, a su vez, oxida la lignina (**Figura 24**) (Call y Mücke 1997).

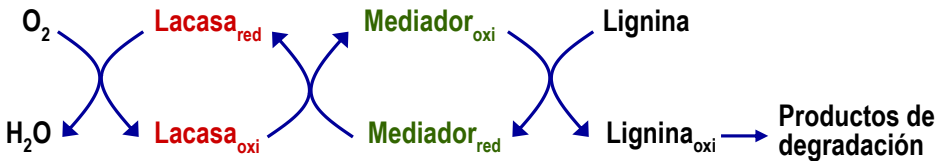


Figura 24. Mecanismo de actuación del sistema lacasa-mediador

Recientemente, se ha demostrado también la gran eficacia del sistema lacasa-mediador en la eliminación de extraíbles lipofílicos de pastas de coníferas, frondosas así como de fibras no madereras (Gutiérrez *et al.* 2006a, 2006b). En estos estudios, la lacasa del basidiomiceto *Pycnoporus cinnabarinus* en presencia del mediador HBT fue muy eficaz en la eliminación de esteroles libres y conjugados (disminución del 95-100%) en pastas kraft de eucalipto; de triglicéridos, ácidos resínicos y esteroles (disminución del 65-100%) en pastas TMP de abeto; y de alcoholes grasos, alcanos y esteroles (disminución del 40-100%) en pastas a la sosa de lino (**Figura 25**).

Sin embargo, debido al elevado coste y los posibles problemas de toxicidad de estos mediadores sintéticos (que dificultan su aplicación industrial), se han dirigido las investigaciones recientemente a la búsqueda de mediadores alternativos (mediadores naturales), derivados de las unidades de lignina (Gutiérrez *et al*. 2007; Babot *et al.* 2011). Entre los mediadores naturales estudiados se encuentra el siringato de metilo (metil-3,5-dimetoxi-4-hidroxibenzoato) (**Figura 26A**), muy idóneo para la deslignificación (Rico *et al.* 2014), y el siringaldehido (4-hidroxi-3,5-dimetoxibenzaldehido) (**Figura 26B**), adecuado para la eliminación de lípidos (Gutiérrez *et al*. 2007).

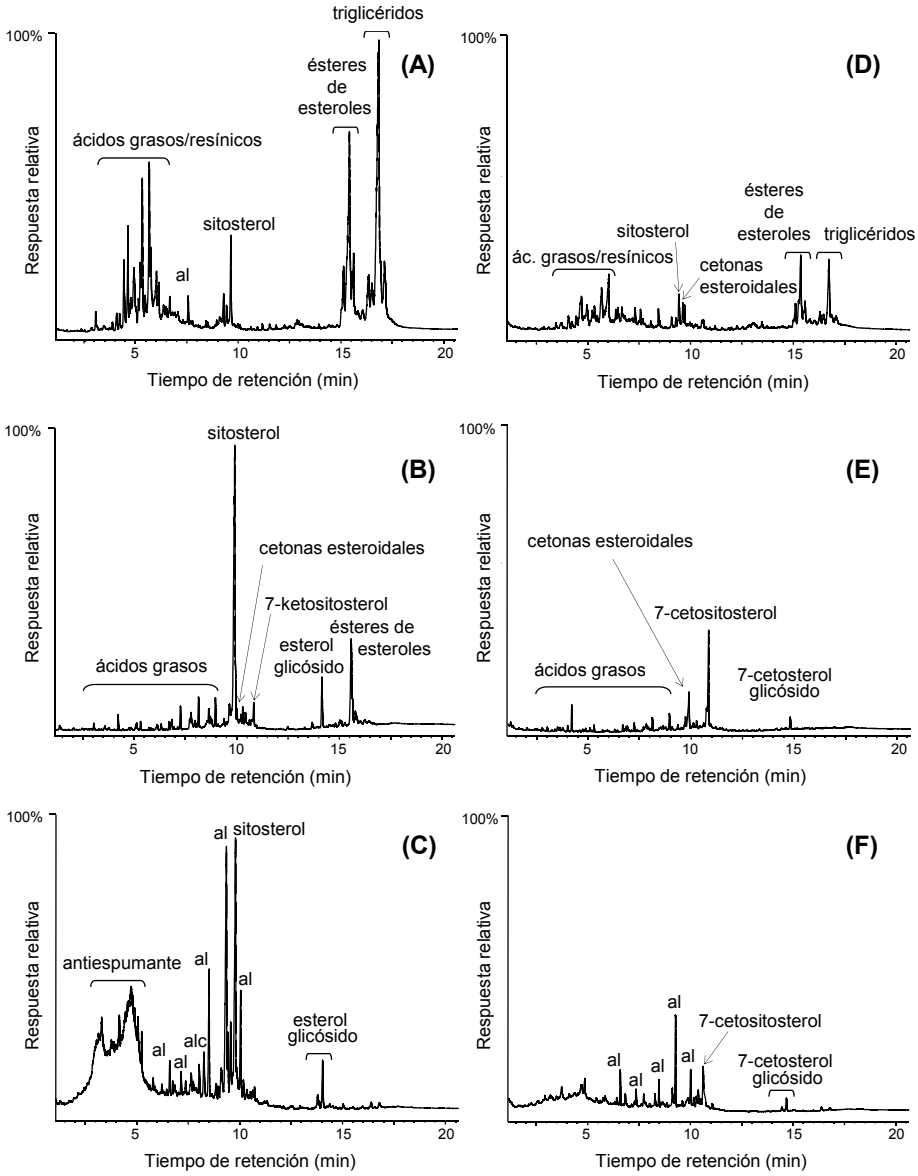
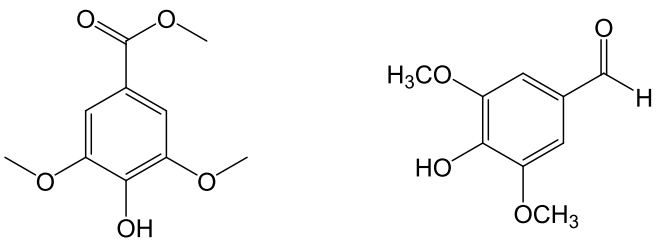


Figura 25. Análisis de cromatografía de gases de compuestos lipofílicos en pasta TMP de abeto (A y D), pasta kraft de eucalipto (B y E), y pasta a la sosa de lino (C y F), antes (A, B y C) y después (D, E y F) del tratamiento con lacasa-HBT. Abreviaturas: *al* alcoholes grasos, y *alc* alcanos. Adaptado de Gutiérrez *et al.* (2006c).



A **B**

Figura 26. Estructura de los mediadores naturales: (A) siringato de metilo y (B) siringaldehido.

Diversos estudios han confirmado el potencial de los sistemas lacasa-mediador para la deslignificación (Poppius-Levlin *et al.* 1999; Ibarra *et al.* 2006; Babot *et al.* 2011), control del *pitch* (Gutiérrez *et al.* 2009), síntesis orgánica (Kunamneni *et al.* 2008), modificación de polímeros (Prasetyo *et al.* 2010), aplicaciones en la industria forestal (Widsten y Kandelbauer 2008) y la producción de bioetanol a partir de pretratamientos físicos/químicos de materiales lignocelulósicos (Palonen y Viikari 2004; Moilanen *et al.* 2011).

En la presente Tesis se ha estudiado la utilización del sistema lacasa-mediador para la deslignificación (y control del *pitch*) de pastas kraft de eucalipto, así como pretratamientos de materiales lignocelulósicos (madereros y no madereros) para la obtención de bioetanol de segunda generación.

La presente Tesis aborda el estudio y desarrollo de tratamientos biotecnológicos aplicados a materiales lignocelulósicos, para obtener un mejor aprovechamiento industrial de los mismos utilizando tecnologías menos contaminantes. Se presta un interés especial a la eliminación/modificación de los extraíbles lipofílicos y al polímero de lignina, ya que ambos constituyentes presentan especial relevancia cuando se utiliza el material lignocelulósico para la producción de pasta y papel, y para la fabricación de biocombustibles de segunda generación como el bioetanol.

Los objetivos específicos de esta Tesis son los siguientes:

- Desarrollar tratamientos enzimáticos basados en el sistema lacasa-mediador que permitan degradar tanto la lignina residual como los compuestos extraíbles lipofílicos presentes en las pastas de celulosa.

- Desarrollar tratamientos enzimáticos basados en el sistema lacasa-mediador que permitan degradar la lignina presente en los materiales lignocelulósicos procedentes de cultivos de crecimiento rápido, tanto madereros (eucalipto) como no madereros (hierba elefante), y así mejorar el rendimiento de la sacarificación enzimática, con vistas a la producción de bioetanol de segunda generación.

- Estudiar las modificaciones estructurales que tienen lugar en el polímero de lignina durante diferentes tratamientos enzimáticos mediante la utilización de la Resonancia Magnética Nuclear bidimensional.

III.1. MATERIALES

III.1.1. Cultivos lignocelulósicos

En la presente Tesis se ha utilizado muestras de eucalipto (*Eucalyptus globulus*) y hierba elefante (*Pennisetum purpureum*).

El eucalipto (*Eucalyptus*) es un género perteneciente a la familia *Myrtaceae*, orden *Myrtale*s, clase *Magnoliopsida* (dicotelidóneas) de la división *Magnoliophyta*. El eucalipto es un árbol de crecimiento rápido. En general, no necesita un suelo con alta fertilidad pero sí con buen drenaje. La especie *Eucalyptus globulus* conocido como gomero azul de Tasmania o eucalipto blanco, es originario del sudeste de Australia y Tasmania. Con una altura comprendida generalmente entre 30-55 m y una anchura de hasta 2 m de diámetro, es una de las especies con más interés industrial, porque su madera tiene una densidad alta (550 kg/m^3), con bajo contenido en lignina y compuestos extraíbles. La madera de eucalipto (*E. globulus*) utilizada en esta Tesis fue suministrada por la empresa papelera ENCE (Pontevedra, España).

La hierba elefante (*P. purpureum*) es una planta perteneciente a la familia *Poaceae,* orden *Cyperales*, clase *Liliopsida* (monocotelidóneas) de la división *Magnoliophyta*. No se debe confundir con la especie *Miscanthus giganteus*, también llamada a veces hierba elefante. Este cultivo, con origen en África, ha sido introducido en muchas zonas tropicales y subtropicales. La hierba elefante crece rápidamente, su altura puede alcanzar hasta 3 m, y llega a producir hasta 45 toneladas de biomasa por hectárea anualmente (Woodard y Prine 1993). Por este motivo, la hierba elefante representa un recurso lignocelulósico atractivo, tanto para la producción de bioenergía y biomateriales, como para la producción de papel. La hierba elefante (*P. purpureum)* utilizada en esta Tesis fue suministrada por la Universidad Federal de Viçosa (Brasil).

Tanto el eucalipto (*E. globulus*) como la hierba elefante (*P. purpureum)* se secaron al aire y se molieron en un molino de cuchillas IKA MF10 pasado por un tamiz de malla Nº 100. Después se pulverizaron finamente en un molino planetario Retsch PM100 (Retsch, Haan, Alemania) a 400 rpm (con 5 min de pausa después de cada 5 min de molienda), utilizando un frasco de ágata de 500 mL y unas bolas de ágata (20 x 20 mm). El tiempo total del molino de bolas para las muestras fue de 5 h.

III.1.2. Pastas de celulosa

La pasta kraft cruda (no blanqueada) de eucalipto (*E. globulus*) utilizada en la presente Tesis fue suministrada por ENCE (Pontevedra, España).

La madera de *E. globulus* utilizada en la cocción kraft se obtuvo de árboles de la especie de 12 a 14 años procedentes de las plantaciones de ENCE. La cocción kraft de las astillas se realizó en un digestor Lorentzen & Wettre 165ºC (50 min), con una relación licor/madera de 3.5, 25% de sulfidez y un 16% de álcali activo. Las características particulares de la pasta kraft son: número kappa de 15.5 (~ 2,3% de contenido de lignina), blancura % ISO de 38.5% y viscosidad intrínseca de 1187 mL/g.

III.1.3. Enzimas

Se utilizaron una lacasa comercial del hongo ascomiceto *Myceliophthora thermophila* y una lacasa del hongo basidiomiceto *Trametes villosa*, ambas proporcionadas por Novozymes (Bagsvaerd, Dinamarca), y una lacasa obtenida de una cepa hiperproductora del basidiomiceto *Pycnoporus cinnabarinus*, proporcionada por Beldem (Andenne, Bélgica). Las actividades de cada una de las preparaciones enzimáticas fueron 954U/ml, 204 U/ml y 45 U/ml, respectivamente. Las actividades de las lacasas se valoraron midiendo la oxidación del ácido 2,2-azino-bis (3-etilbenzotiazolin-6-sulfónico) (ABTS) 5mM tamponado con acetato sódico 0.1 M (pH 5) a 24ºC. La formación del radical catiónico del ABTS se midió a 436 nm (ε_{436} = 29300 $M^{-1}cm^{-1}$). Una unidad actividad enzimática se define como la cantidad de enzima que transforma 1 µmol de ABTS por minuto.

III.1.4. Mediadores

Los compuestos utilizados como mediadores de la lacasa fueron el mediador sintético HBT (1-hidroxibenzotriazol), suministrado por Sigma-Aldrich (Steinheim, Alemania); y los mediadores naturales siringaldehído (4-hidroxi-3,5-dimetoxibenzaldehído), también suministrado por Sigma-Aldrich (Steinheim, Alemania), y siringato de metilo (metil-3,5-dimetoxi-4-hidroxibenzoato), suministrado por Alfa Aesar (Karlsruhe, Alemania).

III.2. MÉTODOS ANALÍTICOS

III.2.1. Evaluación de las propiedades de las pastas

Determinación de la blancura y de la viscosidad intrínseca

Las blancura y la viscosidad intrínseca de las pastas se estimaron mediante los métodos estándar ISO (International Organization for Standardization Documentation and Information 2003) 3688:1999 y

5351/1:1981, respectivamente. Estas medidas fueron realizadas en ENCE (Pontevedra, España).

Determinación del índice kappa

El procedimiento estándar utilizado en la industria para determinar el grado de deslignificación en una pasta química es la determinación del índice kappa por la norma TAPPI T 236 cm-85 (Tappi 2006) y consiste en el volumen en mL de una disolución de $KMnO_4$ 0,1 N consumido por 1 g de pasta. La lignina de la pasta reacciona con el permanganato y la cuantificación del permanganato consumido se determina por titulación con tiosulfato de sodio.

Con ayuda de una batidora de mano, se desintegraron los gramos de pasta necesarios para el ensayo (**Tabla 2**) en 140 mL de agua destilada y se lavó el pie de la batidora con 50 mL de agua. Con agitación constante, se adicionó una mezcla de 25 mL de $KMnO_4$ 0,1 N y 25 mL de H_2SO_4 0,2 N. Al cabo de 5 min se midió la temperatura y después de 10 min se paró la reacción con 5 mL de KI 1,0 N, se colocaron unas gotas de solución indicadora de almidón al 0,2% y se valoró el I_2 liberado con una disolución de $Na_2S_2O_3$ 0,2 N. Previamente, se realizó siempre un ensayo en blanco que no contenía pasta.

El índice kappa se calculó por la expresión:

$$IK = \frac{p \times f}{w}\left[1 + 0{,}013(25 - t)\right] \qquad p = \frac{(b - a)N}{0{,}1}$$

IK: índice kappa

p: volumen de permanganato de potasio 0,1 N consumido en el ensayo (mL)

f: factor de corrección para un consumo de 50% de permanganato de potasio y que depende de p (**Tabla 3**)

w: peso de pasta seca (g)

b: volumen consumido de tiosulfato de sodio para determinación del blanco (mL)

a: volumen consumido de tiosulfato de sodio para determinación de la muestra (mL)

N: normalidad de la disolución de tiosulfato de sodio

t: temperatura del medio de reacción (ºC)

Tabla 2. Peso de pasta ideal (c/ ≈ 7,5% humedad) para la determinación del índice kappa. Adaptado de un documento realizado por Armindo Gaspar de la Universidad de Aveiro.

IK	peso ideal/g	peso seco/g
70,0	0,190	0,176
65,0	0,204	0,189
60,0	0,221	0,205
55,0	0,242	0,224
50,0	0,266	0,246
45,0	0,295	0,273
40,0	0,332	0,308
35,0	0,380	0,351
30,0	0,443	0,410
25,0	0,531	0,492
20,0	0,664	0,615
19,0	0,699	0,647
18,0	0,738	0,683
17,0	0,781	0,724
16,0	0,830	0,769
15,0	0,886	0,820
14,0	0,949	0,879
13,0	1,022	0,946
12,0	1,107	1,025
11,0	1,208	1,118
10,0	1,328	1,230
9,0	1,476	1,367
8,0	1,661	1,538
7,0	1,898	1,757
6,0	2,214	2,050
5,0	2,657	2,460
4,0	3,321	3,705
3,0	4,428	4,100
2,0	6,642	6,150
1,0	13,284	12,300

Tabla 3. Factores f de corrección del consumo de permanganato usado en la determinación del índice kappa (Tappi 2006).

f+	0	1	2	3	4	5	6	7	8	9
30	0,958	0,960	0,962	0,964	0,966	0,968	0,970	0,973	0,975	0,997
40	0,979	0,981	0,983	0,985	0,987	0,989	0,991	0,994	0,996	0,998
50	1,000	1,002	1,004	1,006	1,099	1,011	1,013	1,015	1,017	1,019
60	1,002	1,024	1,026	1,028	1,030	1,033	1,035	1,037	1,039	1,042
70	1,004									

III.2.2. Aislamiento y análisis de los compuestos lipofílicos

El análisis de los compuestos extraíbles lipofílicos de las pastas de celulosa requiere su aislamiento previo. Para ello, se extrajeron con acetona en un extractor de tipo Soxhlet durante 8 h. A continuación se evaporó el disolvente a sequedad en un rotavapor y la cantidad de extracto se determinó por gravimetría. Los extractos lipofílicos obtenidos se redisolvieron en $CHCl_3$ para su posterior análisis por cromatografía de gases (GC) y cromatografía de gases/espectrometría de masas (GC/MS).

En el análisis de lípidos mediante GC y GC/MS, las características de las columnas cromatográficas utilizadas fueron las adecuadas para poder separar e identificar compuestos de alto peso molecular como ceras, ésteres de esteroles, triglicéridos, etc. Previamente se habían realizado estudios sobre procedimientos para el análisis de los extractos lipofílicos de maderas por GC y GC/MS (Gutiérrez *et al*. 1998, 2004) donde se usaron columnas cortas. En estos estudios, las columnas capilares seleccionadas para el análisis de lípidos por GC fueron de longitud corta (de 5 m) ya que proporcionan una conveniente elución y separación de lípidos de alto peso molecular (Wakeham y Frew 1982; Lusby *et al*. 1984; Evershed *et al*. 1989; Sitholé *et al*. 1992; Örså y Holmbom 1994), además de que el análisis se realiza en un corto período de tiempo (20 min). En nuestro caso, los análisis cromatográficos de los extractos se llevaron a cabo en un cromatógrafo de gases Agilent 6890N equipado con un detector de ionización de llama (FID) y una columna capilar DB-5HT (J&W; 5 m x 0.25 mm ID y 0.1 µm de espesor de película). El programa de calentamiento del horno comenzó a 100°C (1 min), seguido de un incremento de temperatura hasta 350°C (3 min) a 15ºC/min. Las temperaturas del inyector y del detector se mantuvieron a 300°C y 350°C, respectivamente. El gas portador que se utilizó fue Helio y la inyección se realizó en modo splitless.

En el caso de los análisis por GC/MS, los cromatogramas obtenidos tienen que ser reproducibles con los obtenidos por GC usando columnas

capilares de 5 m para identificar los diferentes compuestos. No obstante, en el sistema GC/MS, debido a las condiciones de alto vacío a las que opera, no se pueden usar columnas tan cortas, utilizándose normalmente columnas de 10-15 m. En nuestro caso, el análisis mediante GC/MS se llevó a cabo en un cromatógrafo de gases Varian Star 3800 acoplado a un detector de trampa de iones (ITD, Varian Saturn 4000), usando una columna capilar DB-5HT (J&W; 12 m x 0,25 mm ID, con espesor de película de 0,1 μm). El horno se calentó de 120°C (1 min) a 380°C (5 min) a 10ºC/min. La línea de transferencia se mantuvo a 300ºC. La temperatura del inyector se programó de 120ºC (0,1 min) a 380°C con una rampa de 200ºC/min y manteniéndose hasta el final del análisis. El gas portador utilizado fue Helio. La identidad de cada componente se determinó por comparación de sus espectros de masas con los espectros existentes en las librerías (Wiley y NIST) y con espectros publicados anteriormente, por sus fragmentaciones y, cuando fue posible, por comparación con patrones.

III.2.3. Aislamiento enzimático de la lignina

Para obtener una mayor comprensión de la modificación de la estructura de la lignina, ésta fue aislada enzimáticamente (CEL) del resto de constituyentes que forman el material lignocelulósico. Para el aislamiento, las muestras secas se extrajeron tres veces con agua y después tres veces con etanol al 80% por sonicación en un baño ultrasónico durante 30 min cada vez. Las preparaciones CEL fueron aisladas por sacarificación de los polisacáridos enzimáticamente como se ha descrito por Chang *et al.* (1975). Se utilizó Cellulysin® celulasa, una preparación de celulasa cruda de *Trichoderma viride* que también contiene actividad hemicelulasa. Su actividad era ≥10,000 FPU/g de peso seco. El material libre de extractos molido con bolas (200 mg) fue suspendido en 30 mL de acetato de sodio 20 mM (pH 5) en tubos de centrífuga de 50 mL. Se añadió 7,5 mg de Cellulysin, y la reacción fue incubada a 30ºC durante 48 h. Los sólidos se sedimentaros por centrifugación (8000 rpm, 4ºC, 20 min), y se repitió el proceso con tampón y enzima nueva, tres veces. Por último, el residuo (CEL) se lavó con agua destilada, se recuperó por centrifugación y se liofilizó.

III.2.4. Determinación de la fracción hidrosoluble

El porcentaje de los compuestos hidrosolubles se determinó según la norma Tappi T 207 om-88 (Tappi 2004). Para ello, los cartuchos de los cultivos madereros y no madereros extraídos con acetona, una vez secos, se colocaron en matraces con 100 mL de agua destilada y se tuvieron en un baño a 100ºC durante 3 h, al cabo de las cuales el

extracto se concentró en rotavapor y se secó a 100ºC para su determinación gravimétrica.

III.2.5. Determinación del contenido en cenizas

El contenido en cenizas se determinó mediante la norma Tappi 211 om-85 (Tappi 2004). Para ello se depositaron 200 mg de cada una de las muestras en crisoles de porcelana previamente tarados y se introdujeron en la mufla a 575ºC durante 6 h. Para tararlos se limpiaron con HCl y se introdujeron en la mufla a 575ºC durante 1 h. Después de las 6 h en la mufla, se sacaron los crisoles y se pesaron una vez que alcanzaron la temperatura ambiente. Los contenidos en cenizas se expresaron como porcentajes de la materia prima inicial.

III.2.6. Determinación del contenido en carbohidratos

Determinación de azúcares neutros mediante hidrólisis enzimática

Para la determinación de los azúcares neutros de la hierba elefante y la madera de eucalipto se incubó 30 mg de muestra con 3 mL de tampón citrato de sodio (100 mM, pH 5) y un coctel de enzimas (de Novozymes, Bagsvaerd) con celulasas (Celluclast 1.5L) y β-glucosidasa (Novozym 188) durante 72 h a 45ºC. Para una optimización del método se probaron varias dosis de celulasas (10, 5, 2 y 0.5 FPU/g) y de β-glucosidasa (500, 250, 100 y 25 nkat/g). A continuación, se diluyó la muestra hasta 14,4 mL para tomar 4,8 mL, lo que corresponde aproximadamente a 10 mg del material de partida. Posteriormente, se convierten los monosacáridos en alditoles, para su posterior análisis por GC. Para ello se añaden 200 µL de 2-desoxiglucosa, que actúa como patrón interno. Se pasa 1 mL del hidrolizado con el patrón interno a otro tubo, manteniéndolo en hielo, y se añaden 0,1 mL de NH_3 conteniendo 150 mg/mL de $NaBH_4$. Después de incubarse 1 h a 30ºC se añaden 50 µL de ácido acético glaciar en dos veces, para la descomposición del exceso de $NaBH_4$. Por último tiene lugar la acetilación para convertir los monosacáridos en derivados volátiles (acetatos de alditol) para su análisis por GC. A 0,3 mL de la solución se le añaden 0,45 mL de 1-metilimidazol (catalizador) y 3 mL de anhídrido acético y se incubó 30 min a 30ºC. Después de enfriarse en hielo, se añaden 3,75 mL de agua destilada y 2,5 mL de diclorometano, para extraer los acetatos de alditol. Tras agitar se aspira la fase superior acuosa. Este proceso se repite una vez más con 3 mL de agua destilada y 2 mL de diclorometano, y tres veces más con solo 3 mL de agua destilada. Se evapora el diclorometano, se añade acetona y se vuelve a evaporar y se resuspende en cloroformo. La identificación y cuantificación de los diferentes monosacáridos liberados se llevó a cabo por GC. Se utilizó un cromatógrafo de gases Agilent 6890N equipado con

un detector de ionización de llama (FID) y una columna capilar DB-225 (J&W; 30 m x 0,25 mm ID y 0,15 µm de espesor de película). El programa de calentamiento del horno comenzó a 220ºC (5 min), seguido de un incremento de temperatura hasta 230ºC (5 min) a 2ºC/min. Las temperaturas del inyector y del detector se mantuvieron a 225ºC y 250ºC, respectivamente. El gas portador que se utilizó fue Helio y la inyección se realizó en modo split 1:10. La cuantificación se llevó a cabo mediante la integración de los picos cromatográficos correspondientes a cada monosacárido. Se utilizó glucosa, xilosa y arabinosa como patrones externos para elaborar las curvas de calibración.

Determinación de azúcares neutros mediante hidrólisis ácida

En el caso de la hidrólisis ácida las muestras molidas, libres de compuestos extraíbles, se sometieron a una hidrólisis con H_2SO_4 al 72% (peso/peso), a 30ºC durante 1 h. Posteriormente, la solución se diluyó hasta alcanzar una concentración de 4% H_2SO_4 y se autoclavaron (1 h a 120ºC). A continuación, se filtró la suspensión en un quitasatos con filtro de poro nº 3. El residuo insoluble se utilizó para la determinación de la lignina Klason (ver más adelante) y el residuo soluble obtenido de los primeros 144 mL de las muestras filtradas con agua destilada se utilizó para la determinación de azúcares neutros y de la lignina ácido-soluble (ver más adelante). Continuando con la determinación de los azúcares, se tomaron 4,8 mL del filtrado. A partir de aquí, tiene lugar la reducción, acetilación y la identificación y cuantificación de los monosacáridos liberados de la misma manera que la hidrólisis enzimática, con la única diferencia que en la reducción además de añadirse 0,1 mL de NH_3 conteniendo 150 mg/mL de $NaBH_4$ también se añadieron 0,2 mL de NH_3 25% para neutralizar.

III.2.7. Determinación del contenido en lignina

El contenido en lignina total se determinó como la suma del contenido de la lignina ácido-soluble y la lignina ácido insoluble (lignina Klason).

Lignina Klason

El contenido en lignina de la hierba elefante y la madera de eucalipto se determinó por el método Klason según la norma Tappi T222 om-88 (Tappi 2004), con algunas modificaciones. En este método, las muestras molidas, libres de compuestos extraíbles, se sometieron a una hidrólisis con H_2SO_4 al 72% (peso/peso), a 30ºC durante 1 h. Posteriormente, la solución se diluyó hasta alcanzar una concentración de 4% H_2SO_4 y se autoclavaron (1 h a 120ºC). A continuación, se filtró la suspensión en un quitasatos con filtro de poro nº 3. El residuo insoluble (lignina Klason) se

continuó lavando hasta obtener un filtrado de pH neutro y se secó para su cuantificación gravimétrica.

Lignina ácido-soluble

Para la determinación del contenido en lignina ácido-soluble (LAS) se midió la absorbancia del hidrolizado soluble obtenido de la hidrólisis ácida en el análisis de la lignina Klason, a una longitud de onda de 205 nm. Para el blanco se preparó una solución de 4% H_2SO_4. En caso de absorbancias mayores a 1.0 AU se diluyeron los hidrolizados y el blanco apropiadamente.

La lignina ácido-soluble se calculó por la expresión:

$$LAS = \frac{A \times V \times f}{1100 \times m}$$

LAS: lignina ácido-soluble

A: absorbancia a 205 nm

V: volumen final del hidrolizado (mL)

F: factor de dilución

1100: coeficiente de extinción

m: peso de la muestra (g)

III.2.8. Análisis de la lignina

Py-GC/MS

La pirólisis acoplada al cromatógrafo de gases y espectrometría de masas (Py-GC/MS) es un método degradativo que transforma compuestos complejos no volátiles en una mezcla de fragmentos volátiles por descomposición térmica en ausencia de oxígeno (Meier y Faix 1992; Fullerton y Franich 1983). En la pirólisis se producen roturas de los enlaces por acción del calor, ya que cuando la energía aplicada a la molécula es mayor que la energía de enlaces específicos ocurre la disociación de éstos de una forma predecible y reproducible, pudiendo obtener cierta información en relación a la molécula original a través del análisis de los productos de degradación formados. Los fragmentos resultantes de la pirólisis se pueden separar por GC e identificar por MS. La Py-GC/MS es un método poderoso para el análisis de materiales lignocelulósicos, especialmente de la lignina, ya que aunque las cadenas laterales de los monómeros de la lignina se destruyan parcialmente, los sustituyentes metoxilos característicos de las diferentes unidades H, G, S

de la lignina permanecen. La lignina se piroliza produciendo una mezcla de compuestos fenólicos que resultan de la rotura no sólo de enlaces éter, sino también de ciertos enlaces C-C, reteniendo estos fenoles las características de sustitución del polímero de lignina y siendo posible por lo tanto identificar los diferentes componentes de ligninas provenientes de unidades H, G y S. La Py-GC/MS presenta diversas ventajas frente a otros métodos degradativos, pues es una técnica analítica rápida que proporciona resultados en apenas un paso, siendo necesaria poca cantidad de muestra y una simple preparación de la misma (secar y pesar). También presenta ventajas frente a los métodos clásicos de análisis de la lignina, pues no es necesario aislar la lignina de la muestra, permitiendo el análisis de la lignina *in situ*.

En la presente Tesis, la pirólisis de las muestras se llevó a cabo en un pirolizador EGA/Py-3030D (Frontier Laboratories Ltd.), que operó a 500ºC durante 10 s, conectado a un equipo de GC/MS (Agilent 7820A) con una columna capilar DB-1701 (longitud 60, diámetro interno 0.25 mm y espesor de película 0.25 µm). La temperatura del horno de GC se programó a 45ºC (4 min), y aumentó hasta 280ºC (10 min) a 4ºC/min. La columna capilar se acopló a un analizador de masas Agilent mass selector 5975 N con la ionización de electrones programado a 70 eV. El flujo del gas portador (He) fue 1 mL/min. Los compuestos de pirolisis se identificaron con la ayuda de los espectros publicados (Faix *et al.* 1990; Ralph y Hatfield 1991).

2D-NMR

La Resonancia Magnética Nuclear (NMR), tanto de ^{1}H como de ^{13}C, es uno de los métodos más empleados dentro de las técnicas no degradativas para analizar la lignina, que ofrece una información en detalle de la estructura de la lignina, incluyendo los diferentes tipos de unidades y los enlaces que se establecen entre ellas (Robert 1992). En la década de los 70 comenzó a desarrollarse la NMR bidimensional (2D-NMR), aunque la extensión de su uso comenzó en los 80; y en la segunda mitad de los años 80 se desarrolló la NMR tridimensional (3D-NMR). Estas técnicas proporcionan una información detallada de la estructura de la lignina y resuelven señales que aparecían solapadas en los espectros monodimensionales (Capanema *et al*. 2001; Ralph *et al*. 2001; Liitiä *et al*. 2003). La 2D-NMR se considera en la actualidad la técnica más potente en el análisis de la estructura de la lignina y a través de ella se han podido identificar nuevas estructuras en el polímero de la lignina, como las dibenzodioxocinas (Karhunen *et al*. 1995) y las espirodienonas (Zhang y Gellerstedt 2001; Zhang *et al*. 2006).

La espectroscopía 2D de correlación heteronuclear de cuanto simple (2D-HSQC) proporciona correlaciones a través de acoplamiento escalar a un enlace entre un protón y el heteronúcleo al que está directamente unido. Los espectros HSQC de lignina se caracterizan por presentar tres

regiones bien diferenciadas: región alifática, región alifática oxigenada y región aromática (**Figura 27**). La región alifática oxigenada es la más importante para el estudio de la estructura de la lignina ya que en esta zona se encuentran los enlaces de la lignina, además de mostrar señales de metoxilos y carbohidratos. La región aromática es la más importante desde el punto de vista de la composición de la lignina, ya que en esta zona aparecen las correlaciones de las distintas unidades H, G y S.

Para el análisis de los materiales "in situ" por NMR, se disolvieron 100-150 mg de madera de eucalipto o hierba elefante finamente molida en tubos de 5 mm de NMR, y se disolvieron en 0,75 mL de dimetilsulfóxido deuterado (DMSO-d_6), obteniéndose geles. Para el análisis por NMR de la lignina aislada, se disolvieron alrededor de 30 mg en 0,75 mL de DMSO-d_6, disolviéndose totalmente. Las condiciones de adquisición de los espectros HSQC tanto de los materiales "in situ" como de las ligninas fueron idénticas.

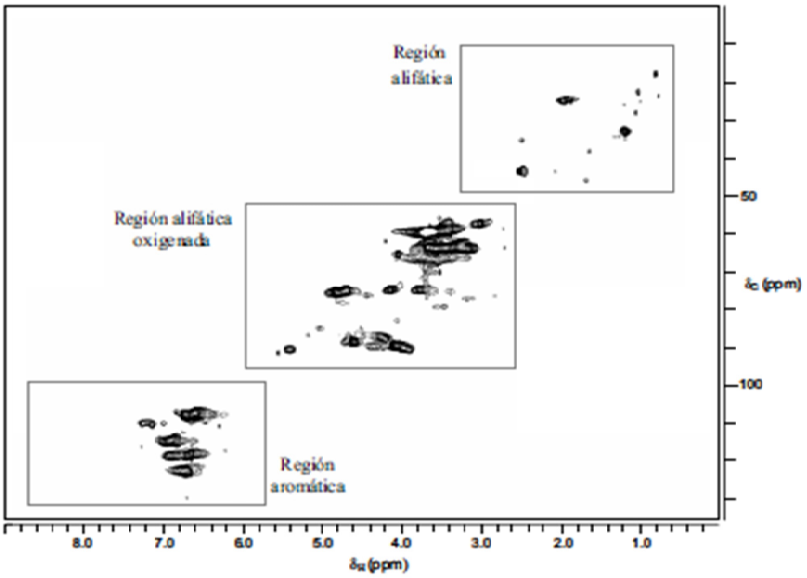


Figura 27. Espectro 2D ^{1}H-^{13}C HSQC de MWL donde se pueden observar sus tres regiones características.

Los espectros 2D ^{1}H-^{13}C HSQC se registraron a 25ºC en un espectrómetro Bruker AVANCE III 500 MHz equipado con una sonda criogénica TCI, de triple resonancia, con geometría inversa (bobina de protones más cercana a la muestra) y gradiente en el eje z. Los

espectros de correlación ^{1}H-^{13}C bidimensional se llevaron a cabo utilizando un programa de pulso HSQC con módulo de incremento de la sensibilidad y pulsos adiabáticos (secuencia de pulsos estándar de Bruker 'hsqcetgpsisp2.2') y los siguientes parámetros: una ventana espectral entre 0 y 10 ppm (5000 Hz) en F2 (^{1}H) utilizando 1000 puntos de datos para un tiempo de adquisición de 100 ms, un tiempo de espera (delay) de relajación de 1 s, y una ventana espectral de 0 a 200 ppm (25,168 Hz) en F1 (^{13}C) utilizando 256 incrementos de 32 scans, para un tiempo de adquisición total de 2 h 34 min. Los experimentos se optimizaron para un valor de la constante de acoplamineto $^{1}J_{CH}$ de 145 Hz. La intensidad de las señales en los espectros HSQC dependen del valor de esta constante así como del tiempo de relajación T_2 (Zhang y Gellerstedt 2007). Por ello, la integración de las señales se realizó por separado en cada una de las regiones del espectro, utilizando las señales correspondientes a correlaciones ^{1}H-^{13}C químicamente análogas, con constantes de acoplamiento $^{1}J_{CH}$ similares. En la región alifática oxigenada, las abundancias relativas de las diferentes subestructuras de la lignina se estimaron mediante la integración de las correlaciones C_α-H_α. En la región aromática, las correlaciones ^{1}H-^{13}C de las unidades S y G se usaron para estimar las relaciones S/G.

III.3. TRATAMIENTOS ENZIMÁTICOS

III.3.1. Tratamientos enzimáticos de pastas de celulosa

Los tratamientos enzimáticos de pastas de eucalipto se realizaron con 5 g de pasta seca, al 3% de consistencia (peso/peso) en tampón dihidrógeno fosfato sódico 50 mM (pH 6.5) cuando se utiliza la lacasa de *Myceliophthora thermophila* o tampón tartrato 50 mM (pH 4) cuando se utiliza la lacasa de *Pycnoporus cinnabarinus*. En los ensayos con el sistema lacasa-mediador se usó siringato de metilo o siringaldehído. La temperatura fue de 50ºC y el tiempo de reacción de 12 h. Los tratamientos se realizaron en matraces de 500 mL, con burbujeo de oxígeno en un baño térmico con agitación (170 rpm). Algunos tratamientos se realizaron en unos biorreactores de 200 mL (Labomat, Mathis) bajo condiciones más similares a los de aplicación industrial (incluye 10% de consistencia de la pasta y 4 bares de oxígeno a presión). En una etapa posterior, tras lavar las pastas con 1 L de agua, las pastas al 5% de consistencia, se sometieron a una etapa de blanqueo con peróxido, usando H_2O_2 al 3% (peso/peso) y NaOH 1,5% (peso/peso), ambos referidos al peso de la pasta seca, a 90ºC durante 2 h. En los controles, el experimento se sometió a las mismas condiciones de reacción, sin enzima ni mediador.

III.3.2. Tratamientos enzimáticos de hierba elefante y madera de eucalipto

Los tratamientos enzimáticos de hierba elefante y madera de eucalipto se realizaron con 2-10 g de muestra seca, al 6% de consistencia (peso/peso) en tampón dihidrógeno fosfato sódico 50 mM (pH 6.5) cuando se utiliza la lacasa de *Myceliophthora thermophila* o tampón tartrato 50 mM (pH 4) cuando se utiliza la lacasa de *Trametes villosa.* En los ensayos con el sistema lacasa-mediador se usó siringato de metilo o HBT. La temperatura fue de 50ºC y el tiempo de reacción de 24 h. Los tratamientos se realizaron en biorreactores de 200 mL (Labomat, Mathis) con 2 bares de oxígeno a presión, en un baño térmico con agitación (170 rpm). En una etapa posterior, tras lavar las pastas con 1 L de agua, las pastas al 6% de consistencia, se sometieron a una etapa de extracción alcalina con peróxido, usando H_2O_2 al 3% (peso/peso) y NaOH 1% (peso/peso), ambos referidos al peso de la pasta seca, a 80ºC durante 90 min. Se realizaron 4 ciclos de tratamientos enzima-extracción sucesivos. En los controles, el experimento se sometió a las mismas condiciones de reacción, sin enzima ni mediador.

En la presente Tesis se ha estudiado la capacidad de un sistema enzima-mediador, formado por enzimas de tipo lacasas y mediadores tanto naturales como sintéticos, para la deslignificación y eliminación de lípidos de pastas de celulosa, así como para la deconstrucción enzimática de la pared celular vegetal de materiales lignocelulósicos para mejorar la sacarificación de los carbohidratos (y su posterior fermentación) con vistas a la producción de bioetanol de segunda generación.

IV.1. DESLIGNIFICACIÓN Y ELIMINACIÓN ENZIMÁTICA DE LÍPIDOS EN PASTAS DE CELULOSA USANDO EL SISTEMA LACASA-MEDIADOR

En esta Tesis se ha estudiado la capacidad de la lacasa recombinante de *Myceliophthora thermophila* (MtL) para deslignificar pastas kraft de eucalipto, y simultáneamente eliminar los componentes lipofílicos de las materias primas causantes de los depósitos de *pitch*. Con este fin se realizaron tratamientos enzimáticos de pasta kraft de eucalipto con MtL (20 U/g) en presencia de dos mediadores fenólicos naturales (siringaldehído, SA y siringato de metilo, MeS), seguidos de una extracción alcalina con peróxido de hidrógeno. A continuación se evaluaron las propiedades de las pastas tras los tratamientos enzimáticos (antes y después de la extracción alcalina) evidenciándose una mejora de las mismas después de la extracción alcalina. Los mejores resultados se obtuvieron en los tratamientos que incluían MeS como mediador, obteniéndose una deslignificación de hasta el 25% y un aumento de la blancura del 15%. Por otro lado, se consiguió una eliminación simultánea de los compuestos lipofílicos presentes en la pasta de eucalipto (incluyendo esteroles, tanto libres como conjugados en forma de ésteres y de glicósidos), que son los principales responsables de la formación de depósitos de *pitch* durante la fabricación de pasta de papel a partir de madera de eucalipto (Gutiérrez *et al.* 2009). La mayor eliminación de los compuestos lipofílicos se alcanzó en el tratamiento con MtL en presencia de SA como mediador, produciéndose una disminución de hasta el 73% de esteroles libres, del 91% de esterol glicósidos y del 89% de ésteres de esteroles. Los resultados obtenidos son muy prometedores con vistas al posterior escalado de este tratamiento ya que se utiliza un sistema lacasa-mediador compuesto por una lacasa comercial (MtL) así como mediadores naturales de bajo coste económico.

Con el fin de mejorar los resultados anteriores se estudió el efecto de la adición de un surfactante (Tween 20) en el tratamiento enzimático de las pastas de celulosa. El Tween 20 sólo contiene ácidos grasos saturados, a diferencia del Tween 80, que contiene ácidos grasos

insaturados que pueden promover reacciones de peroxidación (Jensen *et al.* 1996). En estos estudios también se utilizó Tween 80 con fines comparativos ya que la mayoría de los estudios publicados sobre el uso del sistema lacasa-mediador en pastas de papel se han realizado en presencia de Tween 80 (Moldes y Vidal 2008; Fillat y Roncero 2010a; Fillat *et al.* 2010b). En cuanto las propiedades de las pastas tras la adición de Tween 20 y Tween 80, no se observaron diferencias significativas en la deslignificación entre los tratamientos enzimáticos realizados en ausencia y presencia de Tween. Sólo se observó una mejora en los valores de viscosidad de dichas pastas en los tratamientos con Tween 80 pero fueron acompañados de valores de blancura más bajos. También se evaluó el efecto de la adición de Tween en la eliminación de compuestos lipofílicos en la pasta de eucalipto. En general, la eliminación de sustancias lipofílicas no mejoró con respecto a aquellos tratamientos en los que no hubo adición de Tween. Sólo la eliminación de esteril glicósidos aumentó por la adición de Tween 20 y Tween 80 en los tratamientos con lacasa-MeS (degradación aproximadamente del 50% y 45%, respectivamente).

También se realizaron tratamientos enzimáticos con la lacasa (no comercial) del basidiomiceto *Pycnoporus cinnabarinus* (PcL). Se aplicó a la pasta de eucalipto en presencia de los mediadores SA y MeS. A pesar de que PcL es una lacasa de alto potencial rédox, los resultados de blancura de las pastas tras estos tratamientos fueron similares a los obtenidos con MtL (de bajo potencial redox). Sin embargo, en tratamientos de pasta de eucalipto con PcL en presencia del mediador sintético HBT se obtuvieron mejores resultados de blancura (Camarero *et al.* 2007).

Teniendo en cuenta que los principales obstáculos para la utilización a nivel industrial del sistema lacasa-mediador son los elevados costes de este sistema como consecuencia del uso de altas dosis de enzima y mediador, se evaluaron distintas dosis de enzima y de MeS. Se probaron dosis de MtL entre 1 y 20 U/g de pasta junto con concentraciones de MeS entre 0.6 y 6.7 mM. La **Figura 28** muestra los resultados de blancura y deslignificación (como índice kappa) de las pastas (después de la extracción alcalina con peróxido de hidrógeno) obtenidos utilizando las diferentes dosis de lacasa y mediador. Aunque el mejor resultado de blancura y de índice kappa se obtuvo con la mayor dosis de lacasa y mediador, se observaron resultados muy prometedores a las dosis más bajas de enzima y MeS. Posteriormente se realizaron tratamientos de pasta en biorreactores de 200 mL (Labomat, Mathis), en condiciones parecidas a las de aplicación industrial, incluyendo oxígeno presurizado (4 bares) mientras que los anteriores tratamientos se habían llevado a cabo en matraces con burbujeo de oxígeno. En los tratamientos en biorreactor, se consiguió reducir la duración del tratamiento en más de un

50% con una deslignificación y una mejora de la blancura similar a las obtenidas en matraz, incluso a la dosis más baja de enzima y mediador.

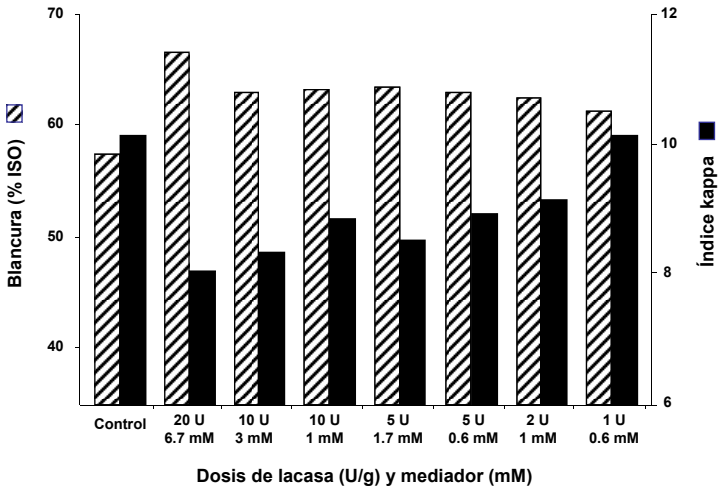


Figura 28. Blancura e índice kappa de la pasta de eucalipto después del tratamiento con diferentes dosis de la lacasa de *M. thermophila* (desde 1 U/g hasta 20 U/g) y metilsiringato (desde 0.6 mM hasta 6.7 mM) seguido de una extracción alcalina con peróxido de hidrógeno, comparado con un control (sin enzima ni mediador).

Cabe mencionar que este tratamiento se realizó a escala piloto en el "Centre Technique du Papier" (Grenoble, Francia), en un estudio en colaboración utilizando la dosis más baja de enzima y mediador. Con esta aplicación se obtuvieron resultados muy positivos, mejorando la calidad de las pastas y modificando todas las propiedades de las mismas positivamente (Burnet *et al.* 2011).

IV.2. PRETRATAMIENTO ENZIMÁTICO DE MADERA DE EUCALIPTO Y HIERBA ELEFANTE CON LA LACASA DE *TRAMETES VILLOSA* Y EL MEDIADOR HBT

En la presente Tesis se estudió por primera vez la capacidad de la lacasa de alto potencial redox de *Trametes villosa* (TvL) combinada con el mediador sintético HBT como pretratamiento de materiales lignocelulósicos madereros, como el eucalipto (*Eucalyptus globulus*), y no madereros, como la hierba elefante (*Pennisetum purpureum*), para eliminar y/o modificar la lignina, con el fin de facilitar la accesibilidad de

las enzimas celulolíticas a los carbohidratos y por tanto mejorar el rendimiento de la sacarificación enzimática con vistas a la producción de bioetanol de segunda generación.

Los pretratamientos se realizaron utilizando dosis diferentes de lacasas (10, 25 y 50 U/g) y una concentración fija (2.5%) del mediador HBT en una secuencia de 4 ciclos que incluía cuatro etapas enzimáticas en presencia de oxígeno seguidas (cada una de ellas) de una extracción alcalina con peróxido de hidrógeno. Tras la secuencia completa se determinó el contenido en lignina (como lignina Klason) de las muestras. Los mejores resultados se obtuvieron con las dosis más altas de enzima, consiguiéndose una eliminación de lignina de la madera de eucalipto de hasta un 48% y de un 32% en la hierba elefante. Por otro lado, se estudió la mejora en la sacarificación enzimática (con celulasas) de la madera de eucalipto y la hierba elefante pretratadas con el sistema lacasa-mediador TvL (25 U/g) y HBT. Las dosis de celulasas utilizadas fueron 10 FPU/g y 500 nkat/g de β-glucosidasa. En el caso de la madera de eucalipto la liberación de glucosa tras la hidrólisis enzimática se estabilizó después de 72 h, con un 61% más de rendimiento respecto al control en ese mismo tiempo. Sin embargo, en el caso de la hierba elefante la estabilización en la liberación de glucosa tuvo lugar tras 4 h de tratamiento con celulasas, aumentando el rendimiento en un 51% respecto al control.

También se realizaron experimentos de sacarificación y fermentación simultánea con estas materias primas, obteniéndose la máxima cantidad de etanol durante las primeras 17 h, aunque se observó una ligera producción del mismo durante el período restante. Aunque en todos los casos los mayores rendimientos (en valor absoluto) de etanol se obtuvieron con la hierba elefante, el tratamiento enzimático fue más eficaz con la madera de eucalipto con una producción mayor de etanol (4 g/L en 17 h) que en el caso de la hierba elefante (2 g/L en 17 h).

Por otro lado, se estudió en detalle la modificación estructural del polímero de lignina de la madera de eucalipto y de la hierba elefante durante los pretratamientos enzimáticos. Para ello, se usó un método desarrollado recientemente en nuestros laboratorios para analizar “in situ” los componentes estructurales de la madera (carbohidratos y lignina) por 2D-NMR (**Figura 29**) y que consiste en disolver el material vegetal finamente molido en dimetilsulfóxido deuterado (DMSO-d_6) formando un gel directamente en el tubo de NMR (Kim *et al.* 2008; Rencoret *et al.* 2009). Las principales subestructuras (enlaces) de lignina identificadas en las muestras de hierba elefante y eucalipto analizadas por HSQC NMR se muestran en la **Figura 30**.

En el caso de la hierba elefante, los análisis de 2D-NMR revelaron un fuerte descenso de los enlaces β-*O*-4′ de la lignina durante los pretratamientos enzimáticos, e incluso su eliminación casi completa a la

dosis más alta de enzima. Del mismo modo, las unidades siringilo (S) de la lignina disminuyeron fuertemente, mientras que aumentaron paralelamente las unidades S oxidadas. Los espectros mostraron una disminución preferente de las unidades guayacilo (G), en comparación con las unidades S, que prácticamente desaparecieron con la dosis más alta de enzima. Sin embargo, las señales de ácido *p*-cumárico permanecieron en el espectro a la dosis más alta de enzima. Por tanto, la lignina de la hierba elefante, que tiene una relación S/G alrededor de 1.2, se enriqueció en unidades S después del tratamiento enzimático. Este resultado fue inesperado, ya que en estudios anteriores de tratamientos de madera de eucalipto con hongos se había observado una disminución en la relación S/G de la lignina tras dichos tratamientos (del Río *et al.* 2002). La eliminación preferente de las unidades G observada en los tratamientos de esta tesis puede deberse a razones topológicas que favorezcan el acceso de los mediadores de la lacasa a las unidades G de la lignina, presentes en los vasos de las plantas (Musha y Goring 1975).

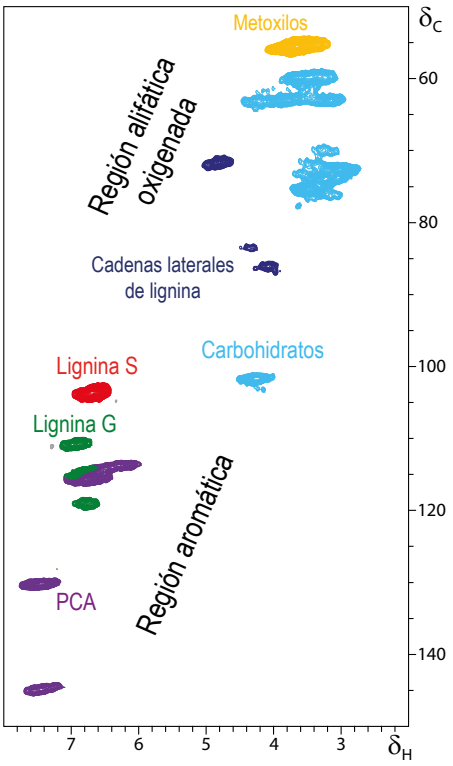


Figura 29. Espectro HSQC NMR de la hierba elefante disuelta en dimetilsulfóxido deuterado mostrando las señales de lignina S y G, y el ácido *p*-cumárico en la región aromática, y las principales señales de enlaces (β-*O*-4′) y carbohidratos en la región alifática (oxigenada).

Por otro lado, los resultados de 2D-NMR de los pretratamientos enzimáticos de madera de eucalipto mostraron la desaparición de los enlaces β-*O*-4′ de la lignina, incluso a las dosis más bajas de enzima. Al igual que ocurría en la hierba elefante, las unidades G de lignina se degradaron preferentemente respecto de las unidades S, llegando a desaparecer completamente, incluso a las dosis más bajas de enzima, mientras la mayor parte de las unidades S se oxidaron. Por tanto, la lignina de la madera de eucalipto tratada enzimáticamente está enriquecida principalmente en unidades S oxidadas, al igual que ocurre durante el tratamiento enzimático de la hierba elefante. La mayor reducción del contenido de lignina de eucalipto con respecto a la hierba elefante durante el pretratamiento enzimático puede ser debida a la mayor relación S/G de la lignina de eucalipto (alrededor de 3.3) de acuerdo con estudios previos (Rencoret *et al.* 2008, 2011).

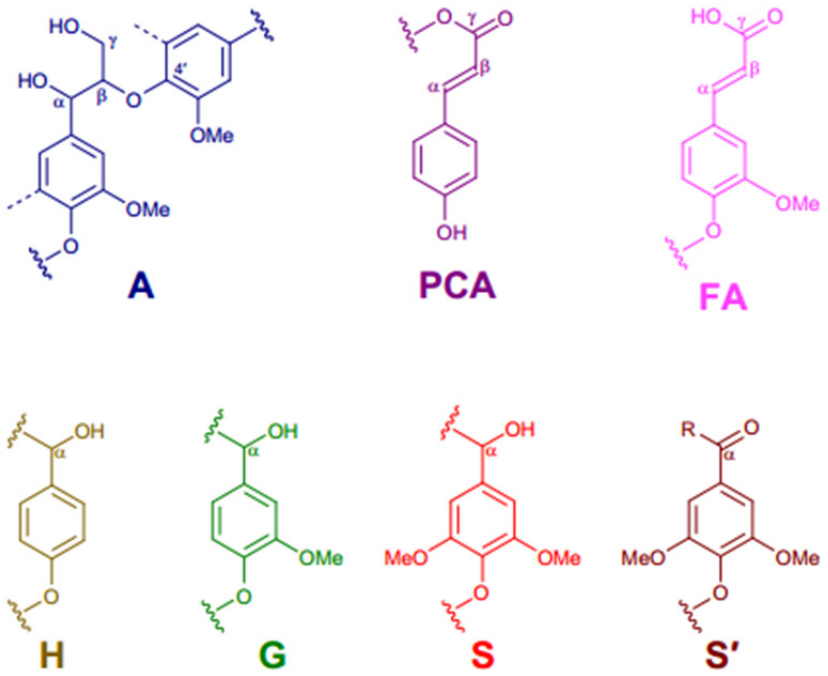


Figura 30. Principales subestructuras (enlaces) de lignina identificadas en las muestras de hierba elefante y eucalipto analizadas por HSQC NMR: (A) subestructuras de lignina β-*O*-4′ (incluyendo una segunda unidad S o G), (PCA) ácido *p*-cumárico, (FA) ácido ferúlico, (H) unidades *p*-hidroxifenilo, (G) unidades guayacilo, (S) unidades siringilo y (S′) unidades S C_{α}-oxidada (R puede ser: OH = ácido terminal, C = cetona, H = aldehído terminal).

IV.3. PRETRATAMIENTO ENZIMÁTICO DE MADERA DE EUCALIPTO CON LA LACASA DE *MYCELIOPHTHORA THERMOPHILA* Y SIRINGATO DE METILO

Los estudios anteriores mostraron el gran potencial del sistema enzima-mediador formado por la lacasa de *T. villosa* y el mediador HBT para la degradación de la lignina en materiales lignocelulósicos madereros y no madereros (Gutiérrez *et al.* 2012). Sin embargo, el elevado coste del HBT dificulta su aplicación industrial, y por eso en la presente Tesis se ha estudiado el uso de mediadores naturales, como el MeS, que muestra resultados prometedores debido a su precio más moderado y a un potencial rédox adecuado (Kulys *et al.* 2002). En estos pretratamientos, realizados con madera de eucalipto, se utilizó la lacasa de *M. thermophila (*MtL*)* (50 U/g) y MeS (3%) como mediador, y a continuación se realizó una extracción alcalina con peróxido en una secuencia de 4 ciclos. Los pretratamientos enzimáticos incluyeron cuatro etapas enzimáticas en presencia de oxígeno (etapas 1, 3, 5 y 7) seguidas (cada una de ellas) de una extracción alcalina con peróxido (etapas 2, 4, 6 y 8). Después de los 4 ciclos se consiguió una disminución en el contenido de lignina de la madera de eucalipto del 50%. Esta disminución en el contenido de lignina consiguió aumentar el rendimiento en la sacarificación enzimática, con una liberación de glucosa de casi el 40%, con respecto a la muestra de madera de eucalipto inicial (utilizando 2 FPU/g de celulasa y 100 nkat/g de β-glucosidasa).

Por otro lado, se estudió en detalle las modificaciones estructurales producidas en la lignina durante las diferentes etapas del tratamiento enzimático mediante 2D-NMR de las muestras en estado de gel (Rencoret *et al.* 2007, 2009, 2011). Los análisis de 2D-NMR revelaron diferencias importantes en las muestras pretratadas enzimáticamente con respecto al control (**Figura 31**). Las principales subestructuras (enlaces) de lignina identificadas en la muestra de eucalipto analizadas por HSQC NMR se muestran en la **Figura 30**. En la etapa 3 (**Figura 31D**) se observó el mayor aumento de la señal de lignina S oxidada. Curiosamente, esta señal disminuyó en todos los casos con la extracción alcalina (etapas 2, 4, 6, 8; **Figuras 31C**, **31E**, **31G**, **31I**). Después de dos ciclos de tratamientos lacasa-mediador y extracción alcalina (etapa 5; **Figura 31F**) se observó la ausencia total de unidades G y de subestructuras de tipo resinol, junto con una alta disminución de enlaces β-*O*-4′ en la lignina. Por último, tras los 4 ciclos de pretratamiento (**Figura 31I**) las señales de los enlaces β-*O*-4′ se redujeron considerablemente con respecto a las señales de carbohidratos y las de unidades S de lignina, destacando la formación de las unidades S oxidadas.

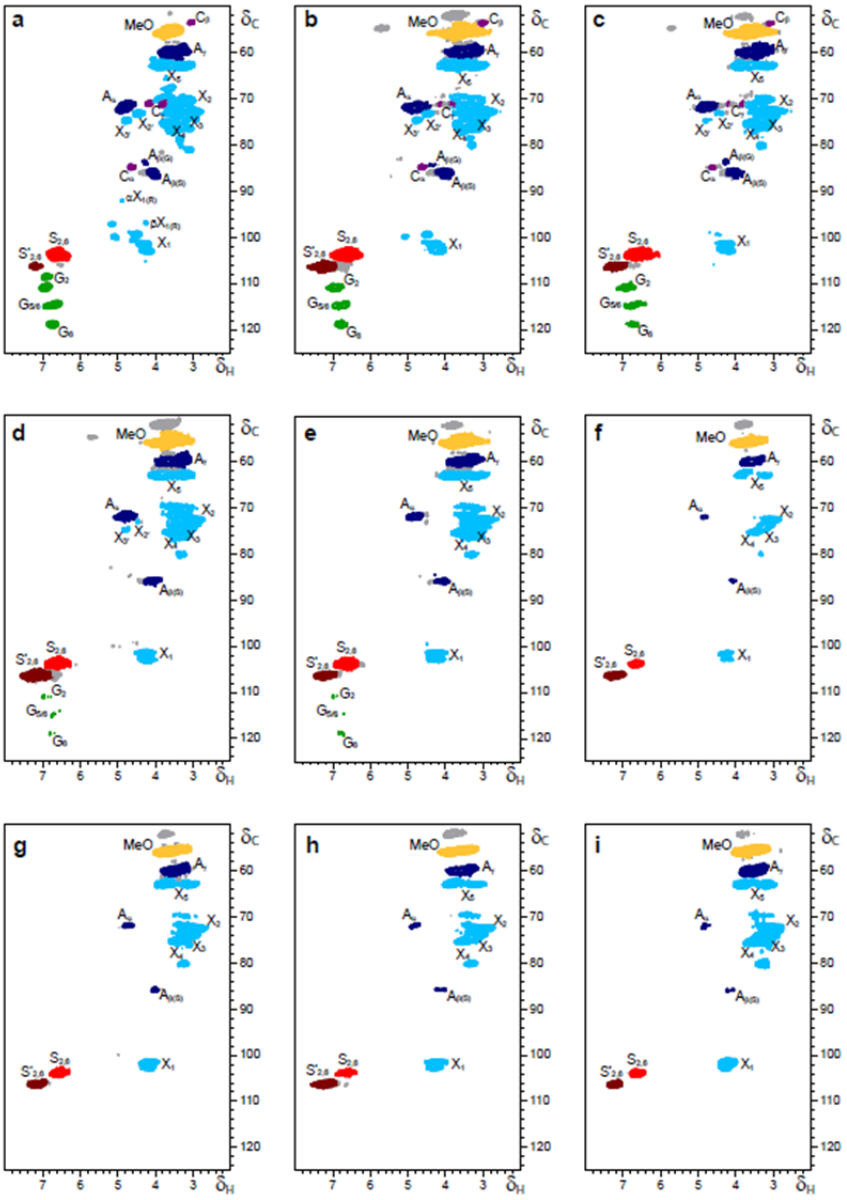


Figura 31. Espectro HSQC NMR de eucalipto (muestras tratadas con la lacasa de *M. thermophila* y MeS) disuelto en dimetilsulfóxido deuterado, con una secuencia enzimática multietapa, incluyendo 4 tratamientos con la lacasa de *M. thermophila* y MeS, y 4 extracciones alcalinas con peróxido: (A) muestra inicial de eucalipto; (B), (D), (F) y (H) muestras del primero, segundo, tercero y cuarto pretratamiento enzimático con lacasa y mediador (etapas 1, 3, 5 y 7, respectivamente); (C), (E), (G) y (I) muestras de la primera, segunda, tercera y cuarta extracción alcalina con peróxido (etapas 2, 4, 6 y 8, respectivamente).

Para obtener un conocimiento más detallado de la modificación de la estructura de la lignina a lo largo de la secuencia del pretratamiento, la lignina de las muestras de eucalipto pretratadas se aislaron enzimáticamente según el método descrito por Chang *et al.* (1975), y se analizaron mediante 2D-NMR. El efecto más notable del pretratamiento con el sistema lacasa-mediador fue la reducción significativa de las unidades de lignina de tipo G, resultando en un incremento de la relación S/G desde 5.0, en la etapa 2, hasta 13.8, en la etapa 8. Además, se produjo un fuerte aumento en la señal de lignina S oxidada a lo largo del tratamiento. Por otro lado, se observó una disminución significativa de enlaces β-*O*-4′ y subestructuras resinol, junto con una disminución menos intensa de las señales de fenilcumarano, espirodienonas y grupos terminales de alcohol cinamílico, que era especialmente evidente en la etapa 8.

Finalmente, se analizaron mediante 2D-NMR los filtrados obtenidos tras cada paso de la secuencia con el objetivo de identificar los productos de degradación de la lignina en la madera de eucalipto. En las muestras tratadas enzimáticamente de las etapas 2 a 8 se observó un aumento de la relación S/G, y la pérdida de subestructuras fenilcumarano, resinol y espirodienonas. También, en comparación con las muestras control y en las últimas etapas, se observó una disminución de los enlaces β-*O*-4′, además de un fuerte incremento en las unidades S oxidadas.

Estos estudios han demostrado por primera vez el gran potencial del uso de una lacasa comercial (de bajo potencial redox), en combinación con mediadores fenólicos naturales, como pretratamiento para deslignificar la madera y mejorar la sacarificación enzimática con vistas a la producción de bioetanol de segunda generación.

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PUBLICACIÓN 1:

Towards industrially feasible delignification and pitch removal by treating paper pulp with *Myceliophthora thermophila* laccase and a phenolic mediator.

Esteban D. Babot, **Alejandro Rico**, Jorge Rencoret, Lisbeth Kalum, Henrik Lund, Javier Romero, José C. del Río, Ángel T. Martínez and Ana Gutiérrez.

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Towards industrially feasible delignification and pitch removal by treating paper pulp with *Myceliophthora thermophila* laccase and a phenolic mediator

Esteban D. Babot[a], Alejandro Rico[a], Jorge Rencoret[a], Lisbeth Kalum[b], Henrik Lund[b], Javier Romero[c], José C. del Río[a], Ángel T. Martínez[d], Ana Gutiérrez[a]

[a]Instituto de Recursos Naturales y Agrobiología de Sevilla, CSIC, PO Box 1052, E-41080, Seville, Spain

[b]Novozymes A/S, Krogshoejvej 36, DK-2880 Bagsvaerd, Denmark

[c]ENCE, Marisma de Lourizán s/n, PO Box 157, E-36080 Pontevedra, Spain

[d]Centro de Investigaciones Biológicas, CSIC, Ramiro de Maeztu 9, E-28040 Madrid, Spain

Abstract

The ability of two natural phenols to act as mediators of the recombinant *Myceliophthora thermophila* laccase (MtL) in eucalypt-pulp delignification was investigated. After alkaline peroxide extraction, the properties of the enzymatically-treated pulps improved with respect to the control. The pulp brightness increased (3.1 points) with MtL alone, but the highest improvements were obtained with syringaldehyde (4.7 points) and especially with methyl syringate (8.3 points) as mediators. Likewise, a decrease in kappa number up to 2.7 points was obtained using methyl syringate, followed by decreases of 1.4 and 0.9 points in treatments with syringaldehyde and MtL alone, respectively. Then, removal of the main lipophilic extractives present in eucalypt pulp was observed after the above laccase-mediator treatments. Finally, the doses of both MtL and methyl syringate were reduced, and results compatible with industrial implementation were obtained.

Keywords: Laccase, Methyl syringate, Pitch deposits, Pulp bleaching, Syringaldehyde

1. Introduction

The paper pulp industry is facing an increasing pressure to replace the conventional pulp bleaching techniques, using chlorine-based chemicals, with more environmentally benign ones. Enzymes offer an environmentally benign and efficient alternative to chemical reagents in many industrial applications, including pulp and paper manufacture (Kenealy and Jeffries, 2003). Lipases (pitch control) and xylanases (bleaching) were introduced in the mill several years ago (Gutiérrez et al., 2001a; Bajpai et al., 2006), yet lignin-degrading oxidoreductases offer additional benefits compared to xylanases for pulp bleaching because they act directly on lignin (Bajpai et al., 2006). Likewise, oxidoreductases also have a high potential for pitch control because they act over a wide range of lipophilic compounds by a recently described oxidative mechanism (Gutiérrez et al., 2009).

Laccases (phenoloxidases, EC 1.10.3.2) are multicopper oxidases that use molecular oxygen as the final electron acceptor and have been object of high interest for the development of environmentally- benign technologies (Mayer and Staples, 2002). The direct action of laccases on lignin is in principle restricted to the phenolic units that only represent a small percentage of the total polymer, a fact that limits its biotechnological application for paper pulp delignification. However, the interest on laccases as industrial biocatalysts steadily increased after discovering the effect on laccase activity of some synthetic compounds, including 2,2'-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) (Bourbonnais and Paice, 1990) and 1-hydroxybenzotriazole (HBT) (Call, 1994) acting as electron carriers between the enzyme and the final substrate. In this way, the action of laccase is expanded to non-phenolic substrates, which are oxidized by the mediator radical, increasing the potential of the enzyme in degradation of lignin and other aromatic compounds. Since then, a variety of studies have confirmed the potential of the so-called laccase-mediator system for bleaching different pulp types (Bourbonnais and Paice, 1996; Poppius-Levlin et al., 1999; Camarero et al., 2004; Moldes and Vidal, 2008; Fillat and Roncero, 2010). On the other hand, the modification of some lipophilic extractives by laccases has been suggested (Zhang et al., 2005), and recently it was reported the high efficiency of the laccase-mediator system for the removal of lipophilic extractives regardless the pulping process and the raw material used (Gutiérrez et al., 2006a; 2006b; Molina et al., 2008; Valls et al., 2009) and a patent application was filed on this application (Gutiérrez et al., 2006c).

However, the cost, safety and environmental profile of some of the synthetic mediators included in most of the above studies make it difficult to implement laccase-mediator systems in pulp bleaching at industrial scale. Recently, several lignin-related natural phenols, which form stable radicals, have been investigated as laccase mediators for pulp bleaching

(Camarero et al., 2007; Fillat et al., 2010b) and removal of lipophilic extractives from pulp (Gutiérrez et al., 2007). The present paper provides the first evidence of the feasibility of using a commercial laccase, and a cheap natural phenol with a suitable redox potential (Kulys et al., 2002) as mediator, for removing lignin and lipids from paper pulp. The enzyme used is the thermostable laccase from the ascomycete *Myceliophthora thermophila*, which has been cloned, expressed in *Aspergillus oryzae*, biochemically characterized, improved for different applications, and commercialized (Xu et al., 1996; Berka et al., 1997). The results presented make the laccase-mediator treatment economically feasible from an industrial point of view.

2. Material and methods

2.1. Pulp

Eucalyptus globulus unbleached kraft pulp, with a kappa number of 15.5 (~2.3% lignin content), brightness of 38.5% ISO, and intrinsic viscosity of 1187 mL g^{-1} was obtained from the ENCE mill in Pontevedra (Spain) and used in enzyme treatment and control experiments.

2.2. Fungal laccase and mediators

A commercial fungal laccase from the ascomycete *M. thermophila,* provided by Novozymes (Bagsvaerd, Denmark) was used in this study. Laccase activity was measured as initial velocity during oxidation of 5 mM ABTS from Roche to its cation radical (ε_{436} 29300 M^{-1} cm^{-1}) in 0.1 M sodium acetate (pH 5) at 24ºC. The laccase activity of the enzyme preparation was 945 U/ml. One activity unit (U) was defined as the amount of enzyme transforming 1 µmol of ABTS per min. A fungal laccase preparation (45 U/ml) obtained from a laccase-hyperproducing strain of the basidiomycete *Pycnoporus cinnabarinus* that was provided by Beldem (Andenne, Belgium) was also used for comparative studies.

Syringaldehyde (4-hydroxy-3,5-dimethoxybenzaldehyde; Cat.: S760-2, Lot.: S16872-326) from Sigma-Aldrich (Steinheim, Germany) and methyl syringate (methyl 4-hydroxy-3,5-dimethoxybenzoate; Cat.: A18054/L05417, Lot.: 10110113) from Alfa Aesar (Karlsruhe, Germany) were assayed as mediators.

2.3. Laccase-mediator treatments of eucalypt pulp

First, pulp treatments with *M. thermophila* laccase-mediator were carried out in duplicate using 10 g (dry weight) of pulp at 3% consistency (w:w) in 50 mM sodium dihydrogen phosphate buffer (pH 6.5). Laccase loading was 20 U g^{-1} pulp and concentration of mediators (syringaldehyde and methyl syringate) in the reaction was kept at 6.75 mM. The treatments were carried out in 500 mL flasks with O_2 bubbling, placed in a thermostatic shaker at 170 rev min^{-1} and 50 ºC, for 12 h. Finally, some enzymatic treatments (with MtL) were performed in 200-mL bioreactors (Labomat, Mathis) under conditions more similar to those of industrial application (including 10% pulp consistency and 4 bar oxygen pressure).

Pulp treatments were repeated in the presence of Tween 20 (Cat.: TW00200250, Lot.: 10926401) or Tween 80 (Cat.: TW00800250, Lot.: 10628801) from Scharlau (0.05% w:v) and with lower doses of laccase (from 20 U g^{-1} to 1 U g^{-1} pulp) and mediator (from 6.75 mM to 0.6 mM).

In a subsequent step, pulps at 5% consistency (w:w) were submitted to an alkaline peroxide extraction (Ep) using 3% (w:w) H_2O_2 and 1.5% (w:w) NaOH, both referred to pulp dry weight, at 90 ºC for 2 h. Controls including laccase without mediator were also performed.

Pulp treatments with *P. cinnabarinus* laccase were performed as described above for *M. thermophila* ones, but using 50 mM sodium tartrate (pH 4) as a buffer.

2.4. Pulp lipid extraction and chromatographic analysis

Treated pulps and controls were air dried and samples were Soxhlet-extracted with acetone for 8 h (Gutiérrez et al., 2001b). All extracts were evaporated to dryness and redissolved in chloroform for analysis of the lipophilic fraction by gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS).

The GC analyses of lipids were performed in an Agilent 6890N Network GC system using a short fused-silica DB-5HT capillary column (5 m x 0.25 mm internal diameter, 0.1 µm film thickness) from J&W Scientific, enabling simultaneous elution of the different lipid classes (Gutiérrez et al., 1998). The temperature program started at 100°C with 1 min hold, then raised to 350°C at 15°C min^{-1}, and held for 3 min. The injector and flame-ionization detector (FID) temperatures were set at 300°C and 350°C, respectively. Helium (5 mL min^{-1}) was used as carrier gas, and the injection was performed in splitless mode. Peaks were quantified by area.

The GC-MS analyses were performed with a Varian 3800 chromatograph coupled to an ion-trap detector (Varian 4000) using a

medium-length (12 m) capillary column of the same characteristics described above for GC using FID. The oven was heated from 120°C (1 min) to 380°C at 10°C min^{-1}, and held for 5 min. The transfer line was kept at 300°C, the injector was programmed from 120°C (0.1 min) to 380°C at 200°C min^{-1} and held until the end of the analysis, and helium was used as carrier gas at a rate of 2 mL min^{-1}. Compounds were identified by mass fragmentography, and by comparing their mass spectra with those of the Wiley and NIST libraries, and standards.

2.5. Py-GC-MS analysis of pulp lignin

Pyrolysis of pulps (approximately 1 mg) after Soxhlet extraction with acetone, was performed with a 2020 micro-furnace pyrolyzer (Frontier Laboratories Ltd.) connected to an Agilent 6890 GC-MS system equipped with a DB-5MS (Agilent J&W) fused-silica capillary column (30 m x 0.25 mm i.d., 0.25 µm film thickness) and an Agilent 5973 mass selective detector (EI at 70 eV). The pyrolysis was performed at 500ºC. The oven temperature was programmed from 40ºC (1 min) to 300ºC at 6ºC min^{-1} (10 min). Helium was the carrier gas (1 mL min^{-1}). The compounds were identified by comparing their mass spectra with those of the Wiley and NIST libraries and reported in the literature (Ralph and Hatfield, 1991).

For pulp lignin analyses in terms of its *p*-hydroxyphenyl:guaiacyl: syringyl (H:G:S) composition, single-ion Py-GC-MS traces were obtained using the molecular ions of the following selected markers (del Río et al., 2001): *m/z* 108 (4-methylphenol), 122 (4-ethylphenol), 120 (4-vinylphenol) and 134 (4-*t*-propenylphenol) for H-lignin units; *m/z* 138 (4-methyl-guaiacol), 152 (4-ethylguaiacol), 150 (4-vinylguaiacol) and 164 (4-*t*-propenylguaiacol) for G-lignin units; and *m/z* 168 (4-methylsyringol), 182 (4-ethylsyringol), 180 (4-vinylsyringol), and 194 (4-*t*-propenylsyringol) for S-lignin units. Phenol, guaiacol and syringol were not selected as lignin markers, to prevent interferences due to eventual formation of syringol from residual amounts of the S-type mediators used.

2.6. Papermaking evaluation of eucalypt pulps

Pulp brightness, kappa number (before and after acetone extraction, as described in section 2.4) and intrinsic viscosity were analyzed following ISO 3688:1999, ISO 302:1981 and ISO 5351/1:1981 standard methods, respectively (International Organisation for Standardization Documentation and Information (ISO), 2003). Data from replicates were averaged. In all cases the standard deviations were below 2% of the mean values.

3. Results and discussion

In this paper, the ability of recombinant *M. thermophila* laccase (MtL) to delignify eucalypt kraft pulp, and simultaneously remove lipophilic extractives causing pitch deposits, in the presence of two simple S-type phenols of plant origin (syringaldehyde and methyl syringate) was tested. The commercially available MtL retains activity through a wide pH range (from pH 4 to pH 9) and has higher thermostability (being active up to 70ºC) than the different basidiomycete laccases investigated for biotechnological application (Xu et al., 1996). More importantly, MtL can be heterologously expressed in industrial hosts (such as *Aspergillus* species) with high yields (Berka et al., 1999), compared with the basidiomycete laccases whose heterologous expression results in low yields currently limiting their large-scale commercialization in pulp bleaching.

3.1. Pulp delignification with MtL and S-type mediators

Unbleached eucalypt kraft pulp was treated with MtL in the presence and absence of syringaldehyde and methyl syringate, and subsequently extracted with alkaline peroxide. The delignification degree was evaluated before and after an alkaline peroxide extraction, by determining the kappa number (an estimation of lignin content in pulp) and the ISO brightness. Intrinsic viscosity (an estimation of cellulose integrity) was also determined. The results obtained are shown in **Table 1**, compared with the control pulp.

Table 1. Pulp papermaking properties (kappa number, brightness, and viscosity) and lignin S/G molar ratio (from selected Py-GC-MS markers, **Fig. 1**) of eucalypt pulp treated with *M. thermophila* laccase (MtL) in the absence and presence of syringaldehyde (SA) or methyl syringate (MS), and control without enzyme, before (initial) and after an alkaline peroxide extraction (Ep).

	Control		MtL		MtL-SA		MtL-MS	
	Before Ep	After Ep	Before Ep	After Ep	Before Ep	After Ep	Before Ep	After Ep
Kappa number	13.3	10.7	12.9	9.8	14.5	9.3	12.2	8.0
Brightness (% ISO)	43.5	57.0	41.5	60.1	35.6	61.7	36.5	65.3
Intrinsic viscosity	1230	1030	1240	1020	1220	972	1240	978
S/G ratio	1.45	0.92	1.10	0.61	1.19	0.55	0.73	0.26

The positive effects of the enzymatic treatments on pulp properties, especially on brightness, were only evidenced after the alkaline extraction, revealing the need of an alkaline peroxide stage after the

treatment with laccase and natural mediators. In the absence of this extraction, the pulp brightness decreased with the enzymatic treatment. Likewise, the kappa number only decreased 0.4 and 1.1 points with the MtL alone and in the presence of methyl syringate, respectively, and increased 1.2 points with the MtL-syringaldehyde treatment (due to partial retention of the mediator on the non-extracted pulp). After the peroxide extraction, the properties of the treated pulps improved with respect to the control. The brightness of eucalypt pulp increased up to 3.1, 4.7 and 8.3 points after the treatments with MtL alone, MtL-syringaldehyde, and MtL-methyl syringate, respectively. On the other hand, a decrease in kappa number up to 2.7 points was obtained after the MtL-methyl syringate treatment followed by a decrease of 1.4 points in the MtL- syringaldehyde treatment and by 0.9 points decrease in the treatment with MtL alone. The increased delignification (up to 25%) and brightness (up to 15%) obtained using methyl syringate as mediator are very promising results since they provide the first evidence on pulp properties improvement using a commercial laccase and a cost-effective natural mediator.

3.2. Comparison with other laccases and mediators

For comparative purposes, the (non-commercial) laccase from the basidiomycete *Pycnoporus cinnabarinus* (PcL) was also applied to eucalypt pulp in the presence of syringaldehyde and methyl syringate as mediators (data not shown). In spite of PcL being a high-redox potential laccase, less reactivity was shown towards lignin (compared with MtL) since worse results in terms of brightness increase (only 0.5 points) and kappa decrease (only 0.1 points) were obtained in the treatments with laccase alone. In the PcL treatments carried out in the presence of syringaldehyde and methyl syringate, similar increases in brightness (about 5 points) and decreases in kappa number (about 1 point) were obtained with both mediators. These results are inferior to those described above using the MtL and methyl syringate as mediator.

The above improvements of eucalypt pulp brightness are lower than reported using the synthetic mediator HBT in combination with high redox-potential laccases from basidiomycetes (Camarero et al., 2007), although the latter treatment has not been industrially implemented due to the high cost and environmental issues associated to this and other synthetic mediators. However, the low redox-potential laccase from the ascomycete *M. thermophila* can be heterologously expressed in *A. oryzae*, in contrast to the high redox-potential basidiomycete laccases. Therefore, MtL has the advantage of being a commercially-available cheap laccase, which will enable the up-scaling of the enzymatic treatment of pulp. Although low redox-potential laccases can not oxidize mediators such as HBT (Ibarra et al., 2006), this is not a problem here since the mediators used (syringaldehyde and methyl syringate) are

phenolic mediators of low redox potential. These phenolic mediators can be easily obtained from natural sources (Tuberoso et al., 2009) and are also present in eucalypt pulping liquor (Gutiérrez et al., 2007). In addition, the decrease in pulp viscosity reported here (5%) is lower than that attained using HBT (18%) (Camarero et al., 2007).

3.3. Enzymatic modification of pulp lignin as shown by analytical pyrolysis

With the aim of investigating how the enzymatic treatment affected the composition of residual lignin, the lignin in the enzymatically-treated pulps was analyzed by pyrolysis coupled to GC-MS. Py-GC-MS allows for *in situ* analysis of lignin by chromatographic separation and mass-spectrometric identification of the compounds released after the pyrolytic breakdown of whole pulp. Unfortunately, the lignin content in chemical pulps is often too low for direct detection of these breakdown products. However, their relative abundances can be analyzed in single-ion chromatographic traces corresponding to selected marker compounds, as described by del Río et al. (2001).

Although the Py-GC-MS analyses were performed after pulp extraction with acetone (and kappa numbers only slightly varied before and after the extraction) the presence of residual syringaldehyde and methyl syringate was detected in chromatographic traces corresponding to their molecular ions (*m/z* 182 and 212, respectively). With the aim of preventing an eventual interference of these compounds, syringol was excluded from the selected markers used to analyze pulp residual lignin, together with phenol and guaiacol (**Fig. 1**). From the traces obtained (that did not include H-type lignin markers), the relative lignin composition in the enzymatically-treated eucalypt pulps and controls were calculated, and shown in **Table 1** as S/G molar ratios (**Table 1**). A slight decrease in the S/G ratio was already observed after the enzymatic treatment with MtL alone, which was the largest after MtL treatment in the presence of methyl syringate followed by the alkaline peroxide stage, indicating a preferential removal of lignin S-units by this laccase-mediator system. This result is in agreement with a more linear structure of S lignin with a predominance of β-O-4' inter-unit linkages (Ibarra et al., 2007), while G lignin is more condensed (branched) due to additional linkages involving the C5 position at the benzenic ring (which is blocked by a second methoxy group in S units) making it more resistant towards degradation.

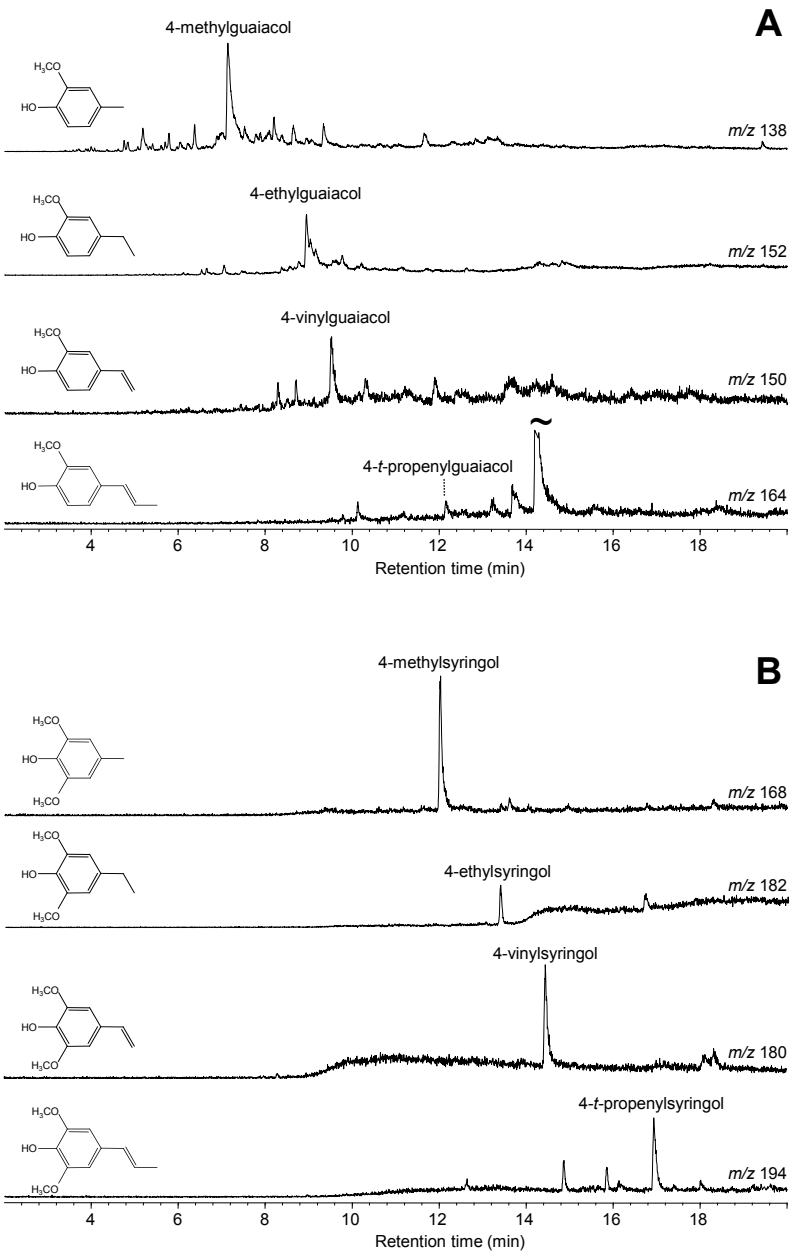

Fig. 1. Single-ion chromatographic traces of markers from Py-GC/MS of paper pulp (control, before alkaline peroxide extraction) used to estimate the lignin S/G ratio. G-lignin markers (**A**) included: 4-Methylguaiacol (*m/z* 138), 4-ethylguaiacol (*m/z* 152), 4-vinylguaiacol (*m/z* 150) and 4-*t*-propenylguaiacol (*m/z* 164). S-lignin markers (**B**) included: 4-Methylsyringol (*m/z* 168), 4-ethylsyringol (*m/z* 182), 4-vinylsyringol (*m/z* 180) and 4-*t*-propenylsyringol (*m/z* 194). Same vertical scale was used in A and B chromatographic traces.

3.4. Pitch removal with MtL and S-type mediators

The removal of lipophilic extractives by eucalypt pulp treatments with MtL and syringadehyde and methyl syringate as mediators, was evaluated before and after the alkaline peroxide extraction by GC and GC-MS analyses. The results obtained are shown in **Table 2**. The main lipophilic compounds present in eucalypt pulp (**Fig. 2A**) included sterols (predominantly sitosterol) in free (**c**) and esterified (**j**) form. Minor amounts of sterol glycosides (**g**) were also present. The detailed composition of lipophilic extractives from eucalypt pulp has been published elsewhere (Gutiérrez and del Río, 2001; 2001b). These compounds have been shown to be the main responsible for pitch problems during manufacturing of eucalypt pulp (Gutiérrez et al., 2009).

Table 2. Removal (percentage of reduction) of the main lipophilic extractives (sterols) from eucalypt pulp after treatment with *M. thermophila* laccase (MtL) in the absence and presence of syringaldehyde (SA) or methyl syringate (MS) before and after an alkaline peroxide extraction (Ep).

	MtL		MtL-SA		MtL-MS	
	Before Ep	After Ep	Before Ep	After Ep	Before Ep	After Ep
Free sterols	25	21	73	69	48	40
Sterol glycosides	0	7	91	86	24	24
Sterol esters	41	41	89	82	92	80

It was observed (**Table 2**) that the enzymatic treatment (without the subsequent alkaline peroxide extraction) using laccase alone was able to partially remove the free sterols (about 25%) and sterol esters (about 41%). When the laccase treatment was performed in the presence of syringaldehyde, a decrease of free sterols (up to 73%), sterol glycosides (up to 91%) and sterol ester (about 89%) contents, was produced (**Fig. 2B**). A similar removal of sterol esters (up to 92%) seems to be attained in the laccase treatments using methyl syringate as mediator (**Fig. 2C**), whereas a lower decrease of free sterols (up to 48%) was observed. A decrease on the sterol glycoside content was not observed with the latter mediator. Similar values of lipophilic extractives removal were obtained in the enzymatic treatments followed by the alkaline peroxide extraction (**Table 2**).

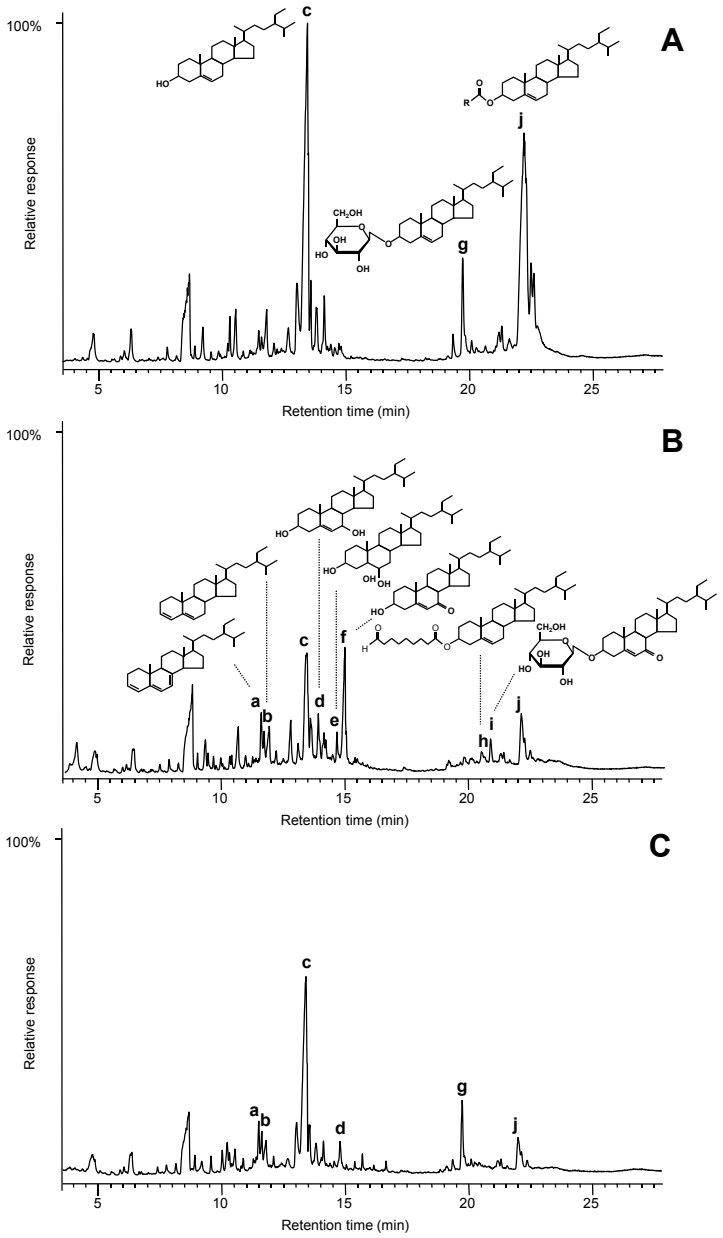

Fig. 2. GC-MS analysis (total-ion chromatograms) and chemical structures of the main lipophilic compounds identified in eucalypt kraft pulp (without alkaline peroxide extraction) before (**A**) and after treatment with laccase *M. thermophila* using syringaldehyde (**B**) and methyl syringate (**C**) as mediators. Peak identification: **(a)** stigmasta-3,5,7-triene; **(b)** stigmasta-3,5-diene; **(c)** sitosterol; **(d)** 7-hydroxysitosterol; **(e)** sitostanetriol; **(f)** 7-ketositosterol; **(g)** sitosteryl 3β-D-glucopyranoside; **(h)** sitosteryl 9-oxononanoate; **(i)** 7-ketositosteryl 3β-D-glucopyranoside; and **(j)** sitosterol esters. Same vertical scale was used in the three chromatograms.

Several oxidation products of steroids were observed in the pulps as a result of enzymatic treatment that were especially evident in the treatments with syringaldehyde (**Fig. 2B**). Some of them were already present in the control although in low amounts. Among them, the main compound identified was 7-ketositosterol (**f**) followed by 7β-hydroxysitosterol (**d**) with only traces of 7α-hydroxysitosterol. Other products found in the treated samples were triols, such as sitostanetriol (**e**). By observing the oxysterol pattern, it could be inferred that oxidation would start with the abstraction of a reactive allylic hydrogen at C_7 followed by oxygen attack to form the peroxy radical. Hydrogen addition to this radical gives the 7-hydroperoxide, whose degradation forms the stable 7α- and 7β-hydroxysitosterol and 7-ketositosterol (Johnsson, 2004). On the other hand, sitostanetriol may be formed after hydration of the epoxides, although the latter compounds were not detected in the pulp samples. Further oxidation of the above oxidation products leads to dehydration and subsequent abstractions of the hydroxy group on C_3, which generates conjugated dienes and trienes such as stigmasta-3,5-diene (**b**) and stigmasta-3,5,7-triene (**a**), which increased in the enzymatically treated pulps. Other oxidized derivatives, which appeared with the enzymatic treatment, included 7-ketositosteryl 3β-D-glucopyranoside (**i**) formed by the oxidation of sitosteryl 3β-D-glucopyranoside and the oxidation products of sterol esters tentatively assigned to sitosterol ester core aldehyde (**n**).

3.5. Effect of Tween (20 and 80) on enzymatic delignification and pitch removal

The above results on pulp delignification and pitch removal were obtained in laccase-mediator treatments without addition of any surfactant. Since most of the published studies on laccase-mediator treatment of paper pulps have been performed in the presence of Tween 80 (Moldes and Vidal, 2008; Fillat and Roncero, 2010; Fillat et al., 2010a), the effect of surfactant addition was also studied here. Taking into account that Tween 80 (due to its content on unsaturated fatty acids) can promote peroxidation reactions (Jensen et al., 1996) in addition to the surfactant effect, enzymatic treatments in the presence of Tween 20 (which contains only saturated fatty acids) were also carried out. **Table 3** shows the effect of the addition of Tween 20 and Tween 80 to the pulp treatments with laccase alone, as well as in the presence of syringaldehyde or methyl syringate, on the pulp properties. Concerning kappa number, no differences could be observed among the enzymatic treatments performed in the absence and presence of Tween. In contrast, lower brightness values were obtained, especially in the presence of Tween 80. Only the viscosity values show an improvement in the treatment with Tween 80.

The effect of adding Tween on the enzymatic removal of lipophilic extractives from eucalypt pulp was also evaluated. Generally, the removal of lipophilic extractives (data not shown) did not improve with respect to those without addition of Tween (**Table 2**). Only the removal of sterol glycosides was increased by the addition of Tween 20 and Tween 80 in the treatments with MtL alone (about 40 and 50% degradation, respectively) and in the presence of methyl syringate (about 50 and 45%, respectively). No differences are observed between the addition of Tween 20 and Tween 80 revealing that only the surfactant effect seems to play a role in this removal.

Table 3. Effect of the addition of Tween 20 (T20) or Tween 80 (T80) on the modification of eucalypt pulp properties (kappa number, ISO brightness and intrinsic viscosity) by *M. thermophila* laccase (MtL) treatment in the absence and presence of syringaldehyde (SA) or methyl syringate (MS), and control without Tween (Con), followed by an alkaline peroxide stage.

	MtL			MtL-SA			MtL-MS		
	Con	T20	T80	Con	T20	T80	Con	T20	T80
Kappa number decrease	0.9	0.8	0.4	1.4	1.4	0.8	2.7	2.4	2
Brightness increase	3.1	2.8	1.1	4.7	4.3	2	8.3	5.9	5
Viscosity decrease	8	13	-38	59	55	-1	53	50	11

3.6. Laccase and mediator doses for pulp delignification and pitch removal

Taking into account that the main obstacles that prevent the use of laccase-mediator systems in the pulp mill are the high costs arisen from the high doses of enzymes and mediators usually reported, therefore, lower enzyme and mediator (methyl syringate) doses have been evaluated here. Laccase dosages between 1 and 20 U per gram of pulp together with mediator doses from 0.6 to 6.7 mM were tested. **Fig. 3** shows the brightness and kappa values (after the alkaline peroxide extraction) obtained using the different doses of laccase and mediator. It is noteworthy that no differences were observed among the treatments using 10, 5 or 2 U g^{-1} of laccase. Likewise, similar brightness results were obtained with methyl syringate doses from 0.6 to 3 mM. The ability of methyl syringate to mediate the oxidation of non-phenolic lignin model compound at low mediator concentration levels has recently been reported (Nousiainen et al., 2009). Although the best brightness and kappa number results were obtained with the highest doses of laccase and mediator, promising results were observed with very low doses of laccase and mediator. Interestingly, when the pulp treatments were performed under oxygen pressure (Labomat experiments under conditions more similar to those of industrial application) the treatment

duration could be reduced over 50% to attain similar kappa and brightness improvements, even with the lowest enzyme and mediator doses.

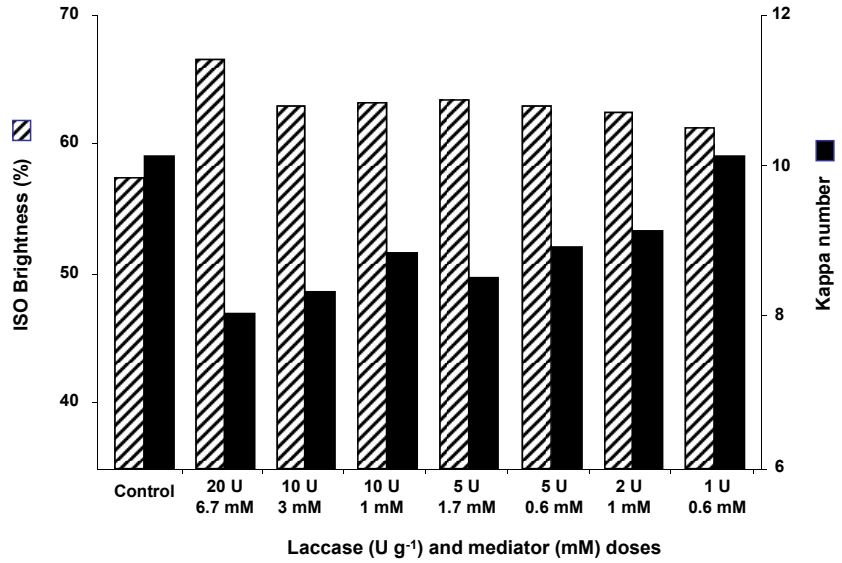

Fig. 3. Brightness and kappa number of eucalypt pulp after treatment with different doses of *M. thermophila* laccase (from 1 to 20 U g^{-1}) and methyl syringate (from 0.6 mM to 6.7 mM) followed by an alkaline peroxide extraction, compared with control (without both laccase and mediator).

The removal of lipophilic extractives from these pulp samples was also evaluated (data not shown). Generally, no differences in the removal of the sterol esters and free sterols were observed among the different treatments and even better results in terms of sterol glycosides removal (54-74% removal) were attained in treatments with low doses of enzyme (1-2 U g^{-1}) and mediator (0.6-1.0 mM).

The combination of substantial bleaching benefits and effective control of pulp extractives giving less down-time may provide sufficient advantages for the enzymatic process to be a cost effective alternative to current bleaching and extractive control programs.

4. Conclusions

In the work reported here, which includes the use of commercial MtL in the presence of lignin-derived phenolic mediators, an important increase of eucalypt pulp brightness and decrease of kappa number were attained using methyl syringate as mediator, together with high removal of pitch-forming lipophilic extractives. Moreover, by careful selection of the laccase-mediator couple low enzyme-mediator doses are able to catalyze pulp delignification and lipid removal thus paving the way for an industrially-feasible cost-effective bleaching stage. This optimized enzymatic stage has potential for substituting oxygen delignification to achieve more sustainable TCF sequences, as suggested by pilot-scale trials currently in course.

Acknowledgements

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PUBLICACIÓN 2:

Demonstration of laccase-based removal of lignin from wood and non-wood plant feedstocks.

Ana Gutiérrez, Jorge Rencoret, Edith M. Cadena, **Alejandro Rico**, Dorothee Barth, José C. del Río and Ángel T. Martínez.

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Demonstration of laccase-based removal of lignin from wood and non-wood plant feedstocks

Ana Gutiérrez[a], Jorge Rencoret[b], Edith M. Cadena[a], Alejandro Rico[a], Dorothee Barth[c], José C. del Río[a], Ángel T. Martínez[d]

[a] Instituto de Recursos Naturales y Agrobiología de Sevilla, CSIC, PO Box 1052, E-41080 Seville, Spain

[b] Department of Biochemistry, Great Lakes Bioenergy Research Center, University of Wisconsin, Madison, Wisconsin 53706, USA

[c] VTT, PO Box 1000, FI-02044 VTT, Finland

[d] Centro de Investigaciones Biológicas, CSIC, Ramiro de Maeztu 9, E-28040 Madrid, Spain

Abstract

The ability of *Trametes villosa* laccase, in conjuction with 1-hydroxybenzotriazole (HBT) as mediator and alkaline extraction, to remove lignin, was demonstrated during treatment of wood (*Eucalyptus globulus*) and non-wood (*Pennisetum purpureum*) feedstocks. At 50 U g^{-1} laccase and 2.5% HBT concentration, 48% and 32% of the *Eucalyptus* and *Pennisetum* lignin were removed, respectively. Two-dimensional nuclear magnetic resonance of the feedstocks, swollen in dimethylsulfoxide-d_6 revealed the removal of *p*-hydroxyphenyl, guaiacyl and syringyl lignin units and aliphatic (mainly β-O-4'-linked) side-chains of lignin, and a moderate removal of *p*-coumaric acid (present in *Pennisetum*) without a substantial change in polysaccharide cross-signals. The enzymatic pretreatment (at 25 U g^{-1}) of *Eucalyptus* and *Pennisetum* feedstocks increased the glucose (by 61% and 12% in 72h) and ethanol (by 4 and 2 g L^{-1} in 17 h) yields from both lignocellulosic materials, respectively, as compared to those without enzyme treatment.

Keywords: Enzymatic deconstruction, Laccase, Bioethanol, Lignocellulose, 2D-NMR

1. Introduction

Lignin removal is an important technical issue for paper manufacturing and a key challenge for the conversion of lignocellulosic feedstock into liquid transportation fuels such as ethanol. Biofuel production from lignocellulosic material requires deconstruction of the cell-wall matrix into individual polymers, and hydrolysis of the carbohydrate polymers into monomeric sugars. Biomass recalcitrance towards enzymatic hydrolysis is correlated with the content and composition of lignin (Studer et al., 2011). Physical, chemical and biological pretreatments, or combinations of these processes, are being studied for deconstructing lignocellulosic biomass and removing lignin (Alvira et al., 2010; Yu et al., 2011). Most biological pretreatments for delignifying lignocellulosic materials employ lignin-degrading fungi, mainly belonging to the group of white-rot basidiomycetes (Kumar et al., 2009; Salvachúa et al., 2011) but such pretreatments require long application periods and consume a fraction of the plant polysaccharides.

Laccases (phenoloxidases, EC 1.10.3.2) are multicopper oxidases that oxidize substituted phenols using molecular oxygen as the final electron acceptor. The direct action of laccases on lignin is, in principle, restricted to phenolic units that only represent a small percentage of the total polymer, a fact that limits their biotechnological application. However, the discovery that some synthetic compounds can act as electron carriers between the enzyme and the final substrate (Bourbonnais and Paice, 1990), 1-hydroxybenzotriazole (HBT) being among the most efficient ones (Call, 1994) has expanded the utility of laccases. Studies have confirmed the potential of laccase-mediator systems for pulp delignification (Poppius-Levlin et al., 1999; Ibarra et al., 2006; Babot et al., 2011), pitch control (Gutiérrez et al., 2009), organic synthesis (Kunamneni et al., 2008), polymer modification (Prasetyo et al., 2010), applications in the forest industry (Widsten and Kandelbauer, 2008) and bioethanol production from physically/chemically pretreated lignocellulose (Palonen and Viikari, 2004; Moilanen et al., 2011).

The present study shows the ability of the high redox-potential laccase from the basidiomycete *Trametes villosa* (Li et al., 1999) to remove lignin and make cellulose accessible to hydrolysis for conversion to fuels, when applied on the whole lignocellulosic biomass in combination with HBT as a redox mediator. Eucalypt (*Eucalyptus globulus*) and Elephant grass (*Pennisetum purpureum*) were selected as representative for rapidly growing, high biomass-producing woody and non-woody plant species, respectively. The modification of lignin in the pretreated lignocellulosic materials was analyzed by two-dimensional nuclear magnetic resonance (2D-NMR) spectroscopy of the whole sample at the gel state (Kim et al., 2008; Rencoret et al., 2009). In addition to lignin removal, the effect of the enzymatic treatments on sugar

and ethanol yield from the two pretreated lignocellulosic materials was also assessed.

2. Material and methods

2.1. Lignocellulosic samples

Elephant grass (*P. purpureum*) from Viçosa Federal University (Brazil) and eucalypt (*E. globulus*) from ENCE (Pontevedra, Spain), were air-dried and ground in an IKA MF10 cutting mill to pass through a 100-mesh screen, and finely ball-milled in a Retsch PM100 ball mill at 400 rev min^{-1} using an agate jar and balls.

2.2. Fungal laccase and mediators

The laccase preparation from the basidiomycete *T. villosa* was provided by Novozymes (Bagsvaerd, Denmark). Its activity was measured as the oxidation of 5 mM 2,2'-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS, from Roche, Mannheim, Germany) to the cation radical (ε_{436} 29300 M^{-1} cm^{-1}) in 0.1 M sodium acetate buffer (pH 5) at 24ºC (Li et al., 1999). One activity unit (U) was defined as the amount of enzyme transforming 1 µmol of ABTS per min. HBT from Sigma-Aldrich (Steinheim, Germany) was used as mediator.

2.3. Laccase-mediator treatments

The eucalypt and Elephant grass samples were treated with the *T. villosa* laccase in the presence (and absence) of HBT, as mediator. Laccase doses of 10, 25 and 50 U g^{-1} were assayed, together with 2.5% HBT (selected after testing several HBT concentrations, from 0.5 to 3%), both with respect to lignocellulosic material dry weight. The HBT concentration was selected after testing several doses (from 0.5 to 3%). The treatments were carried out in 200-mL pressurized bioreactors (Labomat, Mathis) placed in a thermostatic shaker at 170 rev min^{-1} and 50 ºC, using 2 g (dry weight) samples at 6% consistency (w:w) in 50 mM sodium tartrate buffer (pH 4) under O_2 atmosphere (2 bars) for 24 h. After the treatment, the samples were filtered through a Büchner funnel and washed with 1 L of water. In a subsequent step, samples at 6% consistency (w:w) were submitted to a peroxide-reinforced alkaline extraction using 1% (w:w) NaOH and 3% (w:w) H_2O_2 (also with respect to sample dry weight) at 80 ºC for 90 min, followed by water washing (Babot et al., 2011). Cycles of four successive enzyme-extraction treatments were applied. Treatments

with laccase alone (without mediator) and controls without laccase and mediator, were also performed (followed in both cases by the corresponding alkaline extractions).

2.4. Enzymatic hydrolysis

The laccase-pretreated samples were hydrolyzed with a cocktail containing commercial enzymes (from Novozymes, Bagsvaerd) with cellulase (Celluclast 1.5 L; 10 FPU g^{-1}) and β-glucosidase (Novozym 188; 500 nkat g^{-1}) activities, at 1% consistency in 3 mL of 100 mM sodium citrate buffer (pH 5) for 72 h at 45 ºC, with magnetic stirring (in triplicate experiments).

The amount of total sugars released during the enzymatic hydrolyses was determined by the dinitrosalicylic acid (DNS) method (Miller, 1959). The different monosaccharides present were analyzed in a Waters Alliance 2795 high performance liquid chromatography (HPLC) system with an Aminex HPX-87H column (BioRad) and a Waters 2410 refractive index detector, using 5 mM H_2SO_4 (0.6 ml min^{-1}) as eluent. To improve the separation of the monosaccharides, a HPLC Fast Acid Analysis column (BioRad) was included before the above column, and a Cation-H Refill cartridge (BioRad) was added as a pre-column to remove impurities. Glucose, xylose and arabinose were used as standards.

2.5. Fermentation

Simultaneous saccharification and fermentation was conducted at 10% consistency in a 25-mL volume in Erlenmeyer flasks with airlocks (triplicate experiments). The biomass was pre-hydrolysed for 6 h at 45°C as described in section 2.4, RedStar yeast (from Lesaffre, Marcq-en-Barœul, France) was added with an OD_{600} of 3.5 (about 1g L^{-1}), and the flasks were incubated at 30°C with 100 rev·min^{-1} shaking for up to 64 h. The progress of the fermentation was monitored by weighing the flasks regularly, and the ethanol production was calculated from the weight loss.

2.6. Klason lignin content and polysaccharide composition

Klason lignin content was estimated according to T222 om-88 (Tappi, 2006). Monosaccharides in the acid hydrolysate were analyzed by high performance anion exchange chomatography using a CarboPac PA-1 column at 30°C in a Dionex DX 500 series chromatograph equipped with pulse amperometric detection (Dionex ED 40), and expressed in mg of anhydrosugars per 100 mg of sample.

2.6.1. 2D-NMR spectroscopy

Fifty to sixty milligram of lignocellulose samples were swollen in dimethylsulfoxide-d_6 for 2D-NMR at the gel state (Kim et al., 2008; Rencoret et al., 2009). Heteronuclear single quantum correlation (HSQC) 2D-NMR spectra were acquired on a Bruker Biospin (Billerica, MA) AVANCE 500 MHz spectrometer fitted with a cryogenically cooled 5-mm TCI gradient probe with inverse geometry (proton coils closest to the sample). The ^{13}C-^{1}H correlation experiment was an adiabatic HSQC experiment (Bruker standard pulse sequence 'hsqcetgpsisp.2'; phase-sensitive gradient-edited-2D HSQC using adiabatic pulses for inversion and refocusing). Gel HSQC spectra were acquired from 10 to 0 ppm in F2 (^{1}H) with 1000 data points for an acquisition time (AQ) of 100 ms, an interscan delay (D1) of 500 ms, 200 to 0 ppm in F1 (^{13}C) with 400 increments (F1 acquisition time 8 ms) of 40 scans. The $^{1}J_{CH}$ used was 145 Hz. Processing used typical matched Gaussian apodization in ^{1}H and a squared cosine bell in ^{13}C. Prior to Fourier transformation, the data matrices were zero filled up to 1,024 points in the ^{13}C dimension. The central solvent peak was used as an internal reference (δ_C/δ_H 39.5/2.49). The ^{13}C-^{1}H correlation signals of the different lignin units in the aromatic region were used to estimate the lignin composition in terms of *p*-hydroxyphenyl (H), guaiacyl (G), syringyl (S) and oxidized syringyl (S') units, and the *p*-coumaric acid and ferulic acid contents referred to total lignin (H+G+S+S').

3. Results and discussion

3.1. Elephant grass and eucalypt wood delignification with laccase-mediator

The lignin contents (as Klason lignin) of Elephant grass and eucalypt samples after the laccase-mediator sequence were determined and compared with their respective controls (**Table 1**). The lignin content in both lignocellulosic materials decreased considerably, after the enzymatic sequence, concomitantly with increasing laccase doses. For Elephant grass, the decreases were about 11, 22 and 32% of the initial lignin content when using laccase doses of 10, 25 and 50 U/g, respectively (the decreases in lignin content did not stabilize in the course of the enzymatic sequence but progressively increased with respect to the previous step in each of the four laccase-mediator/extraction steps). The reduction in eucalypt wood was more pronounced, attaining 32, 34 and 48% with the above laccase doses. The treatments with laccase alone (without mediator) decreased the lignin content (<5%) in both materials. No significant change in the lignin content, or even a slight increase, have

previously been reported after laccase (alone) treatment of steam pretreated giant read (*Arundo donax*) and spruce (*Picea abies*), respectively (Moilanen et al., 2011). Likewise, no substantial variation in the lignin content and composition (discussed below) was reported after laccase-mediator treatment of steam-exploded eucalypt samples (Martin-Sampedro et al., 2011), most probably because of the different enzyme preparation (Novozym 51003 from Novozymes, Bagsvaerd, based on *Myceliophthora thermophila* laccase) (Li et al., 1999) and treatment conditions used.

Table 1. Lignin content of Elephant grass and eucalypt samples after four enzymatic treatments, each of them followed by an alkaline peroxide extraction, compared with the original untreated material, a control without enzyme and a treatment with laccase alone.

	Elephant grass	Eucalypt
Untreated material	22.1	22.3
Control	21.1	18.0
Laccase (10 U g^{-1}) - HBT	18.8	12.2
Laccase (25 U g^{-1}) - HBT	16.4	11.9
Laccase (50 U g^{-1}) - HBT	14.3	9.4
Laccase (50 U g^{-1})	20.7	17.5

The sugar contents after acid hydrolysis were, glucose (44%), xylose (19%) and arabinose (1%) for untreated Elephant grass, and glucose (44%), xylose (12%), galactose (1%) and mannose (1%) for eucalypt wood. These values were basically the same after treatments with laccase alone, and after treating Elephant grass with laccase-HBT; however, an increase in the glucose (up to 49%) and xylose (up to 13%) contents was observed after treating the eucalypt wood with laccase (25 U g^{-1}) in the presence of HBT, due to the removal of lignin.

3.2. Enzymatic modification of Elephant grass lignin (as shown by 2D-NMR)

Figure 1 shows the complete HSQC NMR spectrum of the whole Elephant grass at the gel state, including the aliphatic oxygenated region, with methoxyl, lignin side-chain and carbohydrate cross-signals, and the aromatic region, with the lignin and *p*-coumaric acid signals (the traces of ferulic acid are not visible in the whole spectrum). The detailed assignments of the different signals are shown in **Fig. 2**, which includes the expanded aliphatic oxygenated (top) and aromatic (bottom) regions of the spectra of the control and laccase-mediator treated samples. The

main lignin and cinnamic structures identified are shown in **Fig. 3**, and the different lignin signals assigned on the spectra are listed in **Table 2**. The lignin composition and *p*-coumaric and ferulic acids contents in the different Elephant grass samples, estimated from the intensities of the corresponding signals in the aromatic region of the HSQC spectra, are shown in **Table 3**.

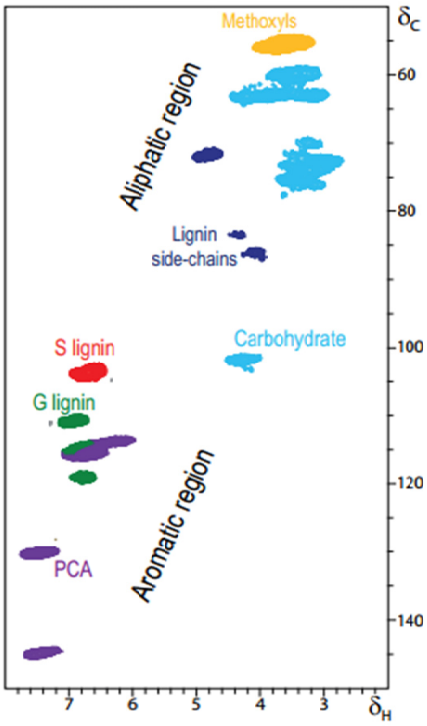

Fig. 1. HSQC NMR spectrum of whole plant biomass (Elephant grass) swollen in dimethylsulfoxide-d_6 showing lignin (G and S units) and *p*-coumaric acid (PCA) signals in the aromatic region, and main lignin inter-unit linkages (β-O-4') and carbohydrate signals in the aliphatic (oxygenated) region. See **Figs. 2** and **5** for detailed signal assignments in Elephant grass and eucalypt samples, respectively, and **Fig. 3** for the main lignin structures identified.

The aliphatic oxygenated region of the spectrum of control Elephant grass (**Fig. 2A**) showed signals of both lignin and carbohydrates. The latter mainly corresponded to xylan (X), since crystalline cellulose is nearly "silent" in lignocellulose gel spectra under solution NMR conditions. In this region, signals of methoxyls and side-chains in β-*O*-4' lignin substructures (A), including C_γ-H_γ, C_β-H_β and C_α-H_α correlations (A_γ,

A_β and A_α, respectively) were observed. The A_γ signal overlaps with related correlations in lignin and other lignocellulose constituents. The C_β-H_β correlations gave two different signals corresponding to β-*O*-4' substructures where the second aromatic unit is a G unit or an S unit ($A_{\beta(S)}$ and $A_{\beta(G)}$, respectively).

The main signals in the unsaturated region of the HSQC spectrum of control Elephant grass (**Fig. 2D**) corresponded to the benzenic rings of the guaiacyl (G) and syringyl (S) lignin units, and the aromatic and olefinic signals of *p*-coumaric acid. The S-lignin units showed a prominent signal for the $C_{2,6}$-$H_{2,6}$ correlation ($S_{2,6}$), while the G-lignin units showed different correlations for C_2-H_2 (G_2), C_5-H_5 (G_5) and C_6-H_6 (G_6). A low intensity signal corresponding to $C_{2,6}$-$H_{2,6}$ correlation in H units ($H_{2,6}$) was also observed. Signals corresponding to $C_{2,6}$-$H_{2,6}$ correlations in C_α-oxidized S-lignin units ($S'_{2,6}$) were hardly observed. On the other hand, the *p*-coumaric acid prominent signals in this region corresponded to the $C_{2,6}$-$H_{2,6}$ ($PCA_{2,6}$) and $C_{3,5}$-$H_{3,5}$ ($PCA_{3,5}$) aromatic correlations, and the C_α-H_α (PCA_α) and C_β-H_β (PCA_β) olefinic correlations. Two low intensity signals corresponding to C_6-H_6 and C_2-H_2 correlations in ferulic acid (FA_6 and FA_2, respectively) were also observed, while the aromatic signals of the ferulic acid traces overlapped with similar signals of *p*-coumaric acid and lignin G units.

The HSQC spectra of the Elephant grass samples after the enzymatic treatments with different laccase doses differed from those of the control (**Fig. 2**). The signals of side-chains in β-*O*-4' lignin substructures (A), present in the aliphatic oxygenated region of the control spectrum, decreased and finally disappeared after the laccase-mediator treatment (**Figs. 2B** and **C**). Likewise, the signal of S lignin units present in the aromatic region of the spectrum also strongly decreased after the laccase-mediator treatment (**Figs. 2E** and **F**), and the signal of C_α-oxidized S-lignin units ($S'_{2,6}$) increased. The enzymatic treatment also enabled detection of new polysaccharide signals corresponding to acetylated xylan (X'), together with a terminal glucose (Gl) signal, that were not detectable in the control samples (probably because of a reduced mobility in the gels due to lignin-hemicellulose linkages). Generation of oxidized lignin structures is congruent with the nature of the lignin biodegradation process, which has been described as an “enzymatic combustion” (Kirk and Farrell, 1987). The action of laccase-HBT on non-phenolic lignin models is produced by hydrogen atom abstraction from the C_α position (Fabbrini et al., 2002). Aromatic ring oxidation, after electron transfer and cation radical formation, has also been reported in model degradation by laccase-HBT, but the C_α attack followed by alkyl-aryl ether breakdown predominates (Kawai et al., 2002). This attack mechanism would result in the increased amount of C_α-oxidized lignin units observed after the laccase-mediator treatment of the Elephant grass, and especially of the eucalypt wood (see below).

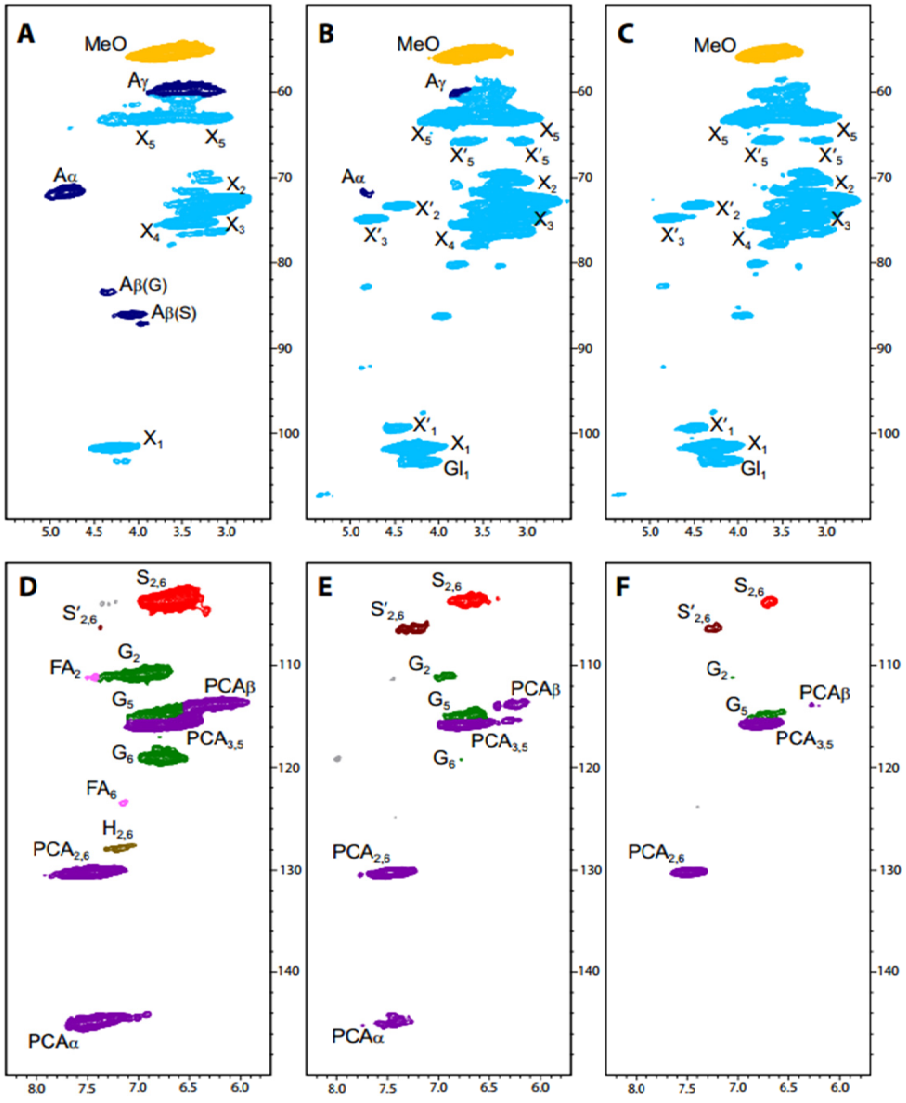

Fig. 2. Expanded aliphatic oxygenated (δ_H-δ_C, 2.5-5.5 and 50-110 ppm; **top**) and aromatic (δ_H-δ_C, 5.7-8.3 and 100-150 ppm; **bottom**) regions of the HSQC NMR spectra of Elephant grass treated with low and high doses of *T. villosa* laccase in the presence of HBT: **A** and **D**) Control without enzyme; **B** and **E**) 10 U·g-1 enzyme; and **C** and **F**) 50 U·g-1 enzyme. See **Table 2** for lignin signal assignment, **Fig. 3** for the main lignin structures identified, and **Table 3** for quantification of these lignin structures. Carbohydrate signals are also observed mainly corresponding to C_1-C_5 in normal (X_1-X_5) and acetylated xylan units (X'_1-X'_5) (an anomeric glucose signal was also identified, G_1).

In the present study, the decrease in G units observed by 2D-NMR, occurred to a greater extent than that of the S ones, and the G units nearly disappeared at the highest laccase dose. Elephant grass lignin which has a similar proportion of S and G units and an S/G ratio around 1.2 in the control sample, became an S-rich lignin after the enzymatic treatments (**Table 3**). This result was unexpected, since fungal treatment of lignocellulosic biomass often cause a decrease in the lignin S/G ratio (del Río et al., 2002), but the result could have been due to topological reasons favoring the access of laccase mediators and/or alkali to the G-rich lignin present in plant vessels (Musha and Goring, 1975). Interestingly, it has been reported that, under alkaline conditions, the very first lignin removed from hardwood is guaiacyl (Santos et al., 2011). In contrast, the most intense signals of *p*-coumaric acid, corresponding to the double aromatic-ring correlations ($PCA_{2,6}$ and $PCA_{3,5}$) remained in the spectrum at the highest laccase dose, although with lower intensities than the carbohydrate signals. The relative molar content of the different lignin units, together with the *p*-coumaric acid content referred to lignin content (PCA/(H+G+S+S') ratio), are shown in **Table 3**, revealing a preferential removal of lignin with respect to *p*-coumaric acid.

Table 2. Assignments of lignin and cinnamic acid main ^{13}C-^{1}H correlation signals in the HSQC NMR spectra of the Elephant grass and eucalypt samples swollen in dimethylsulfoxide-d_6. See **Fig. 3** for chemical structures.

Label	δ_C/δ_H (ppm)	Assignment
MeO	55.6/3.73	C-H in methoxyls
A_γ	59.4 /3.40 and 3.72	$C_\gamma H_\gamma$ in β-*O*-4' structures (**A**)
A_α	71.8/4.83	C_α-H_α in β-*O*-4' structures (**A**)
$A_{\beta(G)}$	83.4/4.27	C_β-H_β in β-*O*-4' structures (**A**) linked to a G-unit
$A_{\beta(S)}$	85.9/4.10	C_β-H_β in β-*O*-4' structures (A) linked to a S unit
$S_{2,6}$	103.8/6.69	C_2-H_2 and C_6-H_6 in syringyl units (**S**)
$S'_{2,6}$	106.1/7.32	C_2-H_2 and C_6-H_6 in α-oxidized syringyl units (**S'**)
FA_2	111.0/7.33	C_2-H_2 in ferulic acid (**FA**)
G_2	110.9/6.99	C_2-H_2 in guaiacyl units (**G**)
PCA_β	113.5/6.27	C_β-H_β in *p*-coumaric acid (**PCA**)
G_5	114.9/6.72 and 6.94	C_5-H_5 in guaiacyl units (**G**)
G_6	118.7/6.77	C_6-H_6 in guaiacyl units (**G**)
FA_6	123.2/7.11	C_6-H_6 in ferulic acid (**FA**)
$H_{2,6}$	127.7/7.20	C_2-H_2 and C_6-H_6 in *p*-hydroxyphenyl units (**H**)
$PCA_{3,5}$	115.5/6.77	C_3-H_3 and C_5-H_5 in *p*-coumaric acid (**PCA**)
$PCA_{2,6}$	130.1/7.45	C_2-H_2 and C_6-H_6 in *p*-coumaric acid (**PCA**)
PCA_α	144.7/7.41	C_α-H_α in *p*-coumaric acid (**PCA**)

A general picture on the compositional changes produced by the enzymatic treatments is provided by **Fig. 4A**, which shows the intensities

of the lignin, *p*-coumaric acid, ferulic acid (traces) and carbohydrate signals in the spectra of the Elephant grass treated with laccase doses of 10, 25 and 50 U g^{-1} in the presence of HBT, and with laccase (50 U g^{-1}) alone, compared with the corresponding control. Although the intensities cannot be used for comparison of different C-H couples in different (aliphatic *vs* aromatic) regions of the same spectrum, they permit a comparison between similar C-H couples in the different samples. In this way, the general tendency observed at increasing enzyme doses is a decrease in lignin carbon and an increase in polysaccharide carbon, in agreement with the chemical analyses. In particular, the decrease in the aromatic carbon in lignin H, G and S units and *p*-coumaric acid, and the aliphatic carbon in lignin side-chains and methoxyls (that also include contributions from hemicelluloses) was observed. In addition, an increase in oxidized S units (relatively moderate in the case of treated Elephant grass) and acylated xylan was observed. In the case of laccase alone, the tendency was the same but the changes observed were relatively minor.

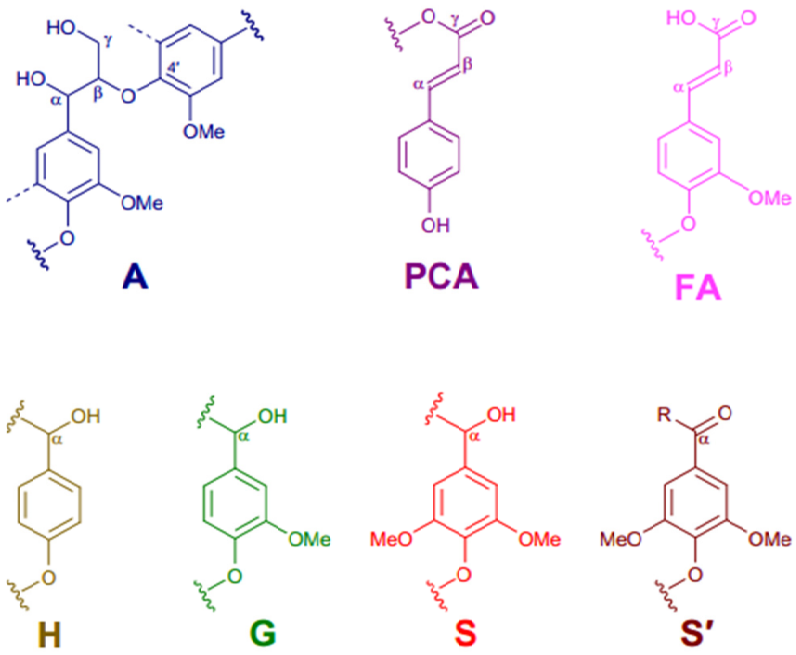

Fig. 3. Main lignin and cinnamic acid structures identified in the Elephant grass and eucalypt samples analyzed by HSQC NMR (**Figs. 1, 2** and **5**): **A**) β-*O*-4' lignin substructures (including a second S or G unit); **PCA**) *p*-coumaric acid; **FA**) ferulic acid; **H**) *p*-hydroxyphenyl units; **G**) guaiacyl units; **S**) syringyl units; and **S')** C_{α}-oxidized S units (R can be a hydroxyl in carboxylic acids or a lignin side-chain in ketones).

3.3. Enzymatic modification of eucalypt lignin (as shown by 2D-NMR)

The detailed assignments of aliphatic-oxygenated (top) and aromatic (bottom) signals in the control and laccase-HBT treated eucalypt samples are shown in the spectra expansions included in **Fig. 5**. The main lignin structures identified are shown in **Fig. 3**, and the different lignin signals assigned on the spectra are listed in **Table 2**. **Table 3** shows the lignin composition in the eucalypt samples, estimated from the intensities of the main cross-signals present in the aromatic region of the NMR spectra.

Table 3. Lignin composition, as molar percentage of H, G, S and C_α-oxidized S units (with respect to total lignin units) and *p*-coumaric acid (PCA) and ferulic acid (FA) content (as cinnamic/lignin molar ratios) from the HSQC NMR spectra of the Elephant grass and eucalypt samples treated with three doses of laccase and HBT (in a sequence including four enzymatic treatments and four alkaline peroxide extractions) compared with a control without enzyme and a treatment with laccase alone.

	H	G	S	S_{ox}	PCA	FA
Elephant grass						
Control	3	43	54	0	0.47	<0.005
Laccase-HBT (10 U g^{-1})	0	34	43	23	0.68	0
Laccase-HBT (25 U g^{-1})	0	30	53	16	0.82	0
Laccase-HBT (50 U g^{-1})	0	16	49	35	0.86	0
Laccase alone (50 U g^{-1})	0	42	58	0	0.66	0
Eucalypt						
Control	0	23	77	0	0	0
Laccase-HBT (10 U g^{-1})	0	0	56	44	0	0
Laccase-HBT (25 U g^{-1})	0	0	41	59	0	0
Laccase-HBT (50 U g^{-1})	0	0	40	60	0	0
Laccase alone (50 U g^{-1})	0	9	91	0	0	0

The aliphatic oxygenated region of the spectrum of control eucalypt (**Fig. 5A**) showed signals of both lignin and carbohydrates, the latter mainly corresponding to xylan units (X), as in the Elephant grass spectra. In addition to methoxyl signals, signals of lignin side-chains were observed with lower intensities than those found in Elephant grass, the latter corresponding to C_α-H_α correlations (A_α) in β-*O*-4' alkyl-aryl ether substructures, and C_β-H_β correlations in β-*O*-4' alkyl-aryl ether substructures including a second S-unit ($A_{\beta(S)}$). The main signals in the aromatic region of the HSQC spectrum of control eucalypt wood (**Fig. 5D**) corresponded to the lignin benzene rings, including the G and S correlations described for the Elephant grass. The content in S units of the eucalypt lignin was higher than that in G units, as revealed by the

prominent $S_{2,6}$ signal, compared with the G_2, G_5 and G_6 signals, with a S/G ratio around 3.3 (**Table 3**), in agreement with previous studies (Rencoret et al., 2008; 2011). The higher reduction in lignin content in eucalypt than in Elephant grass samples could have been due to the higher S/G ratio of eucalypt lignin, which results in a more linear and less condensed polymer.

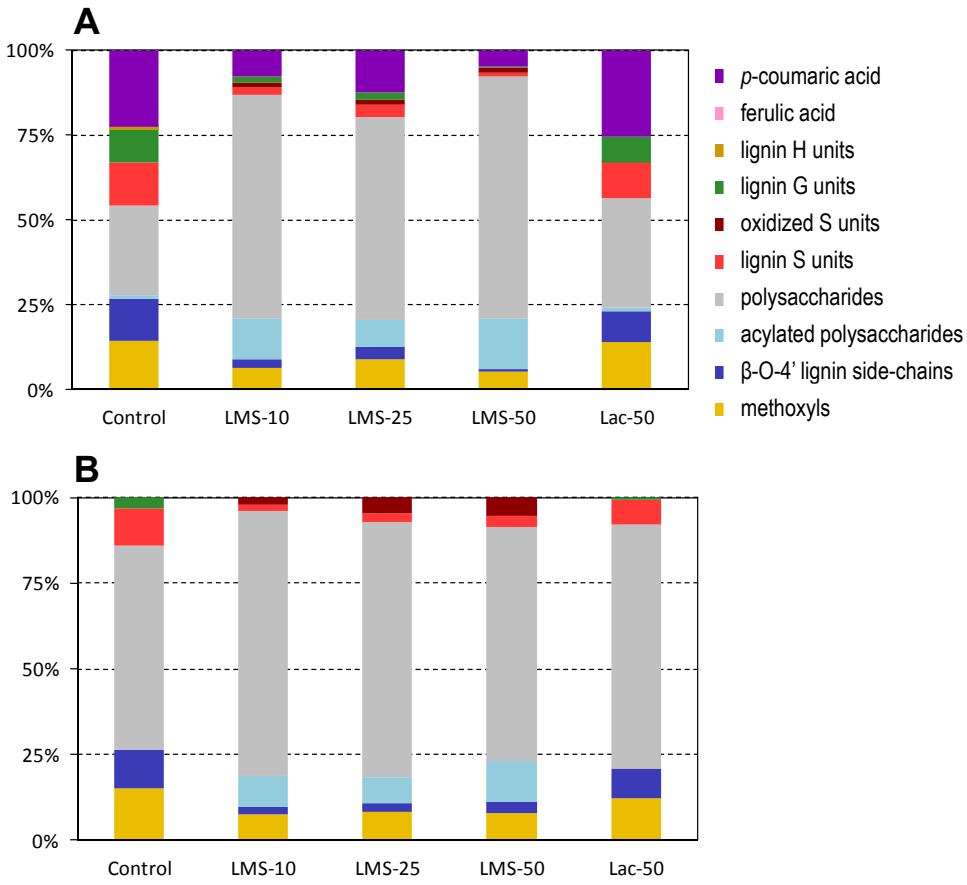

Fig. 4. Comparison of changes in Elephant grass (**A**) and eucalypt (**B**) constituents during laccase-mediator treatment with different enzyme doses (10, 25 and 50 $U \cdot g^{-1}$) as revealed by HSQC NMR, compared with a control without enzyme (first bar) and the treatment with laccase alone (50 U·g-1; last bar). The presence of *p*-coumaric and ferulic acids, H, G, S and S-oxidized lignin, polysaccharide (xylan), acylated polysaccharide, lignin side-chains (in β-*O*-4' substructures) and methoxyl groups was, respectively, shown by the $PCA_{2,6}$, $H_{2,6}$, G_2, $S_{2,6}$, $S'_{2,6}$, X_1, X'_3, A_α and MeO signals in **Figs. 2** and **5** (shown as percentages of sample carbon corresponding to each structure type).

The HSQC spectra of the eucalypt samples treated with laccase-mediator showed important differences compared to the control ones (**Fig. 5**). The signal of side-chains in β-*O*-4' lignin substructures (A_α) present in the aliphatic oxygenated region of the control spectrum completely disappeared even at the lowest enzyme dose (**Figs. 5B**). Likewise, the G lignin signals, in the aromatic region of the spectrum, also completely disappeared with the lowest enzyme dose, while the S units were C_α-oxidized (and in a significant extent remained as such) as revealed by the strong increase in the $S'_{2,6}$ signal, which became the most prominent signal in this region when the enzyme dose was increased (**Fig. 5E** and **F**, and **Table 3**). Therefore, the results obtained for the eucalypt wood confirmed the C_α-oxidation mechanism for lignin removal by laccase-HBT, and revealed that most of the residual lignin in wood treated with the highest laccase dose corresponds to the oxidized S' units. Similarly to the Elephant grass enzymatic treatment, the decrease in G units seemed to occurre to a greater extent than in the S ones. The presence of oxidized lignin units was also observed in eucalypt pulp residual lignin after a laccase-mediator treatment, including both C_α ketones and carboxylic acids (Ibarra et al., 2007).

A general picture of the compositional changes revealed by the NMR analyses of the eucalypt samples, enabling comparison of treatments with different laccase doses (in the presence of HBT) and with laccase alone, is shown in **Fig. 4B**. The general tendency at increasing enzyme doses is a decrease in lignin carbon (in aromatic, side-chain and methoxyl structures), although to a lower extent than in the Elephant grass samples, and a concomitant increase of polysaccharides, including acetylated units. In contrast, the effect of laccase alone was very moderate, being basically reduced to the decrease in lignin G units.

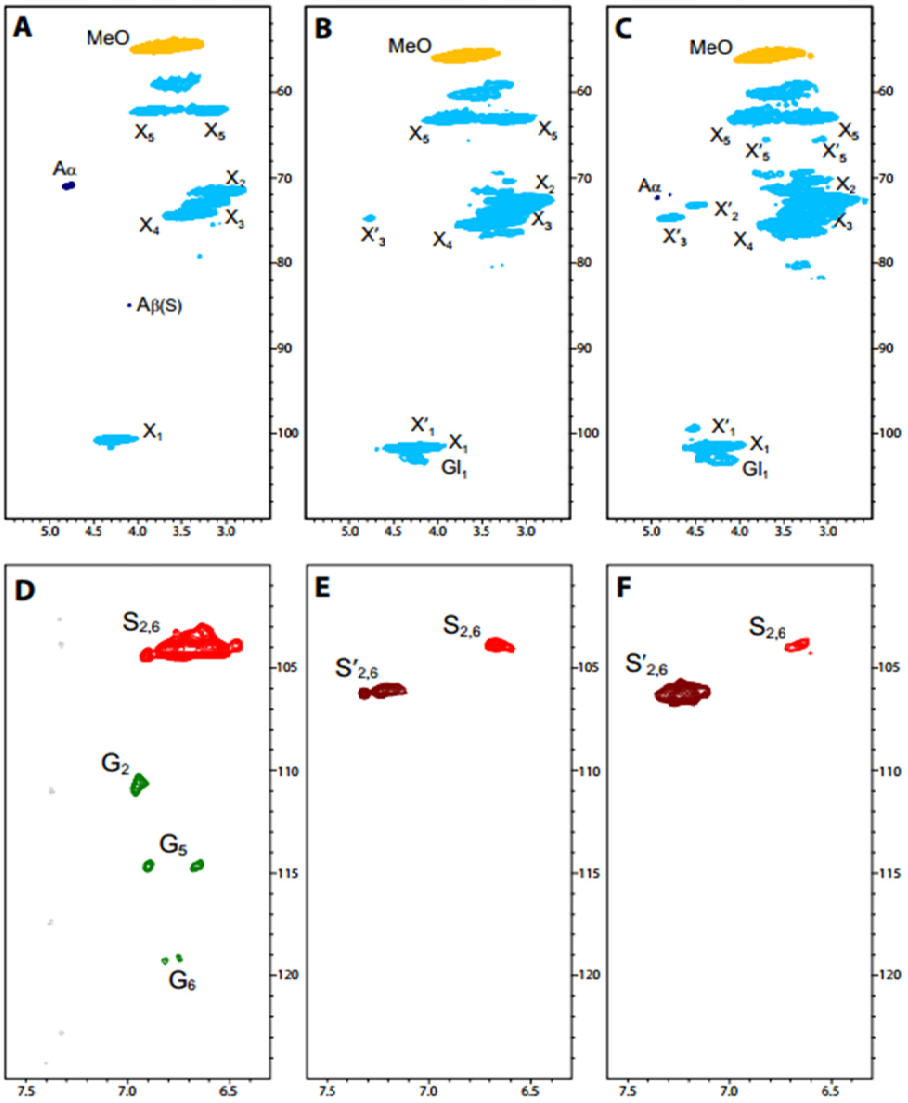

Fig. 5. Expanded aliphatic oxygenated (δ_H-δ_C, 2.5-5.5 and 50-110 ppm; **top**) and aromatic (δ_H-δ_C, 6.3-7.6 and 100-125 ppm; **bottom**) regions of the HSQC NMR spectra of eucalypt treated with low and high doses of *T. villosa* laccase in the presence of HBT: **A** and **D**) Control without enzyme; **B** and **E**) 10 U·g-1 enzyme; and **C** and **F**) 50 U·g-1 enzyme. See **Table 2** for signal assignment, **Fig. 3** for the main lignin structures identified, and **Table 3** for quantification of these lignin structures.

3.4. Enzymatic hydrolysis and fermentation

The Elephant grass and eucalypt samples treated with laccase (25 U g^{-1}), alone and in the presence of HBT, were hydrolyzed using a cellulase and β-glucosidase cocktail, and the main monosaccharides released (glucose, xylose and arabinose) were analyzed by HPLC (total reducing sugars from the DNS assay showed similar tendencies). The effect of hydrolysis time was investigated and 72 h hydrolysis was chosen since monosaccharide release already stabilized after this time period, and reached 64-71% of sample weight for Elephant grass and 35-58% for eucalypt (**Fig. 6A**, **Table 4**). In the case of eucalypt wood, the effect of the laccase-HBT treatment increased with cellulase hydrolysis times, the highest increases in glucose and xylose releases were obtained after 72 h. However, for Elephant grass the highest increases in sugar releases by the laccase-mediator treatment were observed after only a 4-h hydrolysis. The ability of laccase-mediator treatment to increase polysaccharide hydrolysis by cellulases had been already reported, but only on pretreated (steam-exploded) wood (Palonen and Viikari, 2004). Interestingly, the treatment with laccase alone (without mediator) also slightly increased the hydrolysis yields for eucalypt and Elephant grass, with respect to that of the controls. This agrees with the findings by Moilanen et al. (2011) who reported a 12% hydrolysis increase (after 48 h) by laccase (alone) treatment of steam-pretreated spruce wood, although, surprisingly, the same enzymatic treatment on giant read decreased the hydrolysis yield.

During the subsequent long-term simultaneous saccharification and fermentation (**Fig. 6B**), the maximal ethanol production rate (0.32-0.76 g L^{-1} h^{-1}) was achieved during the first 17 h, although further, a slight production was observed during the remaining period (0.02-0.05 g L^{-1} h^{-1}). The latter is explained by the moderate but significant effect caused by milling. In all cases, the highest total ethanol yields were obtained from Elephant grass compared with eucalypt wood. The laccase-mediator pretreatment significantly increased ethanol production after 17 h of saccharification-fermentation. Interestingly, the enzymatic treatment was considerably more efficient improving ethanol production from eucalypt (over 4 g L^{-1} in 17 h) than from Elephant grass (~2 g L^{-1} in 17 h). The presence of the mediator seems necessary to improve ethanol production, since treatment with laccase alone was useless on Elephant grass, and only caused a very moderate increase in ethanol production from eucalypt (0.4 g L^{-1} in 17 h).

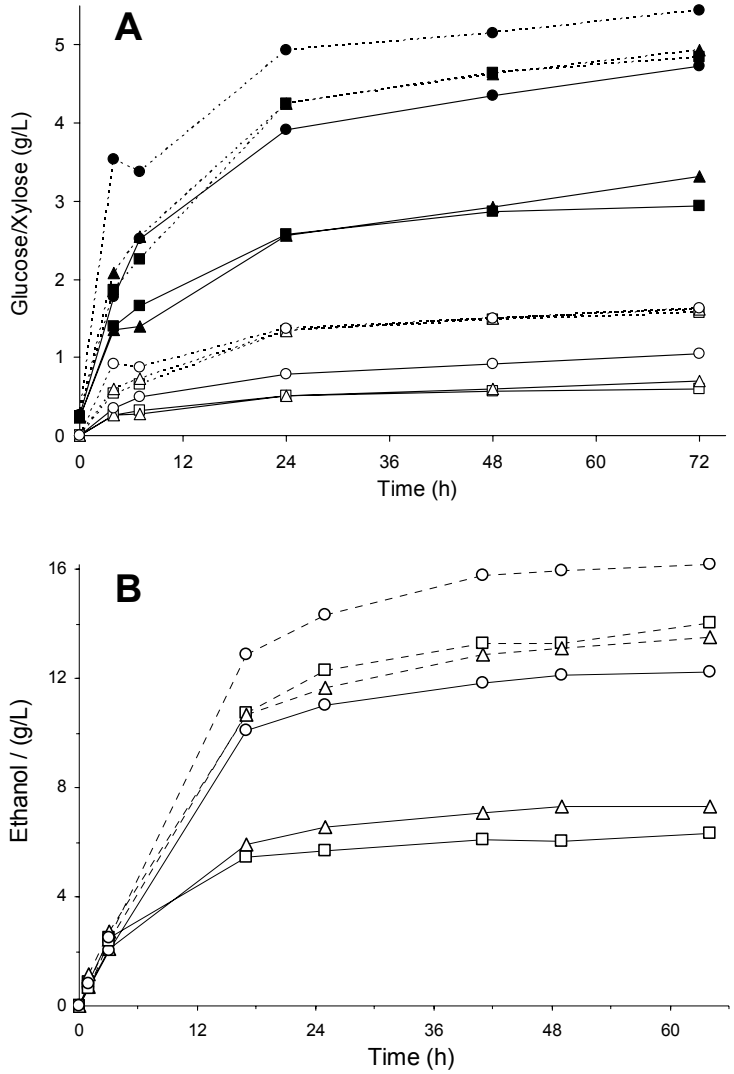

Fig. 6. Effect of enzymatic pretreatment on biomass enzymatic hydrolysis and saccharification: **A)** Comparison of glucose (black symbols) and xylose (white symbols) release during 0-72 h hydrolysis of Elephant grass (dashed lines) and eucalypt (continuous lines) pretreated with laccase-HBT (circles) and laccase alone (triangles) and controls without enzyme (squares); **B)** Time-course of ethanol production during simultaneous saccharification and fermentation of milled Elephant grass (dashed lines) and eucalypt (continuous lines) pretreated with laccase-HBT (circles) and laccase alone (triangles), in sequences including four enzymatic treatments and four alkaline peroxide extractions, and controls without enzyme (squares). Mean values from triplicate experiments. See **Table 4** for final sugar yields and ethanol production.

Table 4. Monosaccharide (glucose and xylose) after 72 h (and 4 h, parenthesis) hydrolysis (% of sample weight), and ethanol release 64 h (and 17 h, parenthesis) after adding the yeast to the cellulase reaction (% of sample weight) from the Elephant grass and eucalypt samples treated with laccase-HBT (25 U g^{-1}) in a sequence including four enzymatic treatments (and four alkaline peroxide extractions) compared with a control without enzyme and a treatment with laccase alone. Means ± S.D (from triplicates).

	Hydrolysis		Fermentation
	Glucose (%)	Xylose (%)	Ethanol (%)
Elephant grass			
Control	48.4 ± 1.3 (18.6 ± 1.3)	15.7 ± 0.8 (5.5 ± 0.4)	14.1 ± 0.1 (10.7 ± 0.2)
Laccase-HBT	54.4 ± 1.3 (35.3 ± 1.6)	16.3 ± 0.6 (9.2 ± 0.5)	16.2 ± 0.6 (12.9 ± 0.1)
Laccase alone	49.4 ± 1.2 (20.8 ± 2.7)	16.1 ± 0.4 (5.9 ± 0.6)	13.6 ± 0.6 (10.7 ± 0.2)
Eucalypt			
Control	29.4 ± 5.1 (14.0 ± 2.6)	6.0 ± 1.0 (2.6 ± 0.4)	6.3 ± 0.4 (5.5 ± 0.2)
Laccase-HBT	47.3 ± 1.5 (17.7 ± 1.1)	10.5 ± 0.2 (3.6 ± 0.2)	12.3 ± 0.2 (10.1 ± 0.1)
Laccase alone	33.1 ± 1.1 (13.5 ± 1.6)	7.0 ± 0.3 (2.6 ± 0.4)	7.3 ± 0.9 (5.9 ± 0.1)

4. Conclusions

Woody and nonwoody plant biomass can be significantly delignified by enzymes (30-50% lignin removal) by applying a sequence consisting of successive laccase-mediator and alkaline extraction stages, directly on the ground lignocellulosic material (i.e. without a partial degradation and subsequent deconstruction). The HSQC NMR spectra of the lignocellulosic samples showed a significant decrease of both aromatic and aliphatic lignin signals after the enzymatic treatments, and provide strong evidence for a C_α-oxidation degradation mechanism, with high presence of oxidized S units in the residual lignin. The improved cellulose hydrolysis, and higher ethanol production in enzyme/mediator-treated feedstock demonstrates the potential of this approach in biofuel production.

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PUBLICACIÓN 3:

Pretreatment with laccase and a phenolic mediator degrades lignin and enhances saccharification of *Eucalyptus* feedstock.

Alejandro Rico, Jorge Rencoret, José C. del Río, Angel T. Martínez and Ana Gutiérrez.

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Pretreatment with laccase and a phenolic mediator degrades lignin and enhances saccharification of *Eucalyptus* feedstock

Alejandro Rico[a], Jorge Rencoret[a], José C. del Río[a], Angel T. Martínez[b], Ana Gutiérrez[a]

[a]Instituto de Recursos Naturales y Agrobiología de Sevilla, CSIC, PO Box 1052, E-41080, Seville, Spain

[b]Centro de Investigaciones Biológicas, CSIC, Ramiro de Maeztu 9, E-28040 Madrid, Spain

Abstract

Background: Biofuel production from lignocellulosic material is hampered by biomass recalcitrance towards enzymatic hydrolysis due to the compact architecture of the plant cell wall and the presence of lignin. The purpose of this work is to study the ability of an industrially available laccase-mediator system to modify and remove lignin during pretreatment of wood (*Eucalyptus globulus*) feedstock, thus improving saccharification, and to analyze the chemical modifications produced in the whole material and especially in the recalcitrant lignin moiety.

Results: Up to 50% lignin removal from ground eucalypt wood was attained by pretreatment with recombinant *Myceliophthora thermophila* laccase and methyl syringate as mediator, followed by alkaline extraction in a multistage sequence. The lignin removal directly correlated with increases (approximately 40%) in glucose and xylose yields after enzymatic hydrolysis. The pretreatment using laccase alone (without mediator) removed up to 20% of lignin from eucalypt wood. Pyrolysis-gas chromatography/mass spectrometry of the pretreated wood revealed modifications of the lignin polymer, as shown by lignin markers with shortened side chains and increased syringyl-to-guaiacyl ratio. Additional information on the chemical modifications produced was obtained by two-dimensional nuclear magnetic resonance of the whole wood swollen in dimethylsulfoxide-d_6. The spectra obtained revealed the removal of guaiacyl and syringyl lignin units, although with a preferential removal of the former, and the lower number of aliphatic side-chains per phenylpropane unit (involved in main β-*O*-4' and β-β' inter-unit linkages), in agreement with the pyrolysis-gas chromatography/mass spectrometry

results, without a substantial change in the wood polysaccharide signals. However, the most noticeable modification observed in the spectra was the formation of C_α-oxidized syringyl lignin units during the enzymatic treatment. Further insight into the modifications of lignin structure, affecting other inter-unit linkages and oxidized structures, was attained by nuclear magnetic resonance of the lignins isolated from the eucalypt feedstock after the enzymatic pretreatments.

Conclusions: This work shows the potential of an oxidative enzymatic pretreatment to delignify and improve cellulase saccharification of a hardwood feedstock (eucalypt wood) when applied directly on the ground lignocellulosic material, and reveals the main chemical changes in the pretreated material, and its recalcitrant lignin moiety, behind the above results.

Keywords: 2D-NMR, Analytical pyrolysis, Bioethanol, *Eucalyptus globulus*, Enzymatic delignification, Laccase, Lignin, Lignocellulose, Pretreatment, Saccharification

1. Background

Lignocellulosic biomass is a renewable resource of great interest for the sustainable production of fuels, materials and chemicals. Among biomass sources, *Eucalyptus* plantations offer a viable feedstock because they are among the fastest growing tree plantations in the world [1]. However, the conversion of lignocellulosic biomass is challenged by its recalcitrant structure. Cellulose, hemicelluloses and lignin are the three main components of lignocellulose, linked into a complex matrix highly resistant to chemical and biological conversion. Biofuel production from lignocellulosic material requires deconstruction of the cell wall into individual polymers, and hydrolysis of the carbohydrates into monomeric sugars. One of the major factors causing biomass recalcitrance towards saccharification is correlated with the content and composition of lignin [2-4].

Lignin is a three-dimensional polymer constituted by phenylpropanoid subunits linked together by a variety of ether and carbon-carbon bonds. Lignin is intimately interlaced with hemicelluloses in the plant cell wall forming a matrix to cover the crystalline cellulose microfibrils. Its aromatic nature and complex structure make lignin degradation very difficult. Both lignin and lignin-derived compounds have a detrimental effect on the hydrolysis of biomass because they physically hinder the accessibility of cellulases; they also bind cellulases and lead to their inactivation [5-9]. Biotechnology can contribute to plant biomass deconstruction by

providing biocatalysts to degrade or modify lignin and lignin-derived compounds [10].

Biomass pretreatment to remove lignin is essential for the enzymatic hydrolysis of lignocellulose. Physical, chemical and biological pretreatments, or combinations of these processes, are being studied for deconstructing lignocellulosic biomass and removing lignin [11-13]. Most biological pretreatments employ lignin-degrading fungi belonging to the group of white-rot basidiomycetes [14,15] but such pretreatments require long application periods and consume a fraction of the plant polysaccharides.

Laccases (phenoloxidases, EC 1.10.3.2) are multicopper oxidases that oxidize substituted phenols using molecular oxygen as the final electron acceptor. The direct action of laccases on lignin is, in principle, restricted to phenolic units, which only represent a small percentage of the total polymer, a fact that limits their biotechnological application. However, the discovery that some synthetic compounds can act as electron carriers between the enzyme and the final substrate [16], 1-hydroxybenzotriazole (HBT) being among the most efficient ones [17], has expanded the utility of laccases. A number of studies have confirmed the potential of laccase-mediator systems for paper pulp delignification [18,19], pitch control [20], polymer modification [21], other applications in the forest industry [22], and bioethanol production from physically and/or chemically pretreated lignocellulose [23]. Recently, the ability of high redox-potential laccases from basidiomycetes of the genus *Trametes* to remove lignin (when applied in combination with HBT) from whole [24] and ensiled [25] lignocellulosic biomass, making cellulose accessible to hydrolysis, was reported. However, most of the studied mediators are synthetic compounds based on nitrogen heterocycles whose high cost and potential toxicity make it difficult to implement laccase-mediator systems at an industrial scale.

Recently, several natural phenols, which form stable aromatic radicals and are available as chemical pulping by-products [26], have been investigated as laccase mediators for pulp biobleaching [27-29] and removal of lipophilic extractives from paper pulp [26]. In the present study, a recombinant laccase from the ascomycete *Myceliophthora thermophila* in combination with the natural mediator methyl syringate was tested for the removal of lignin from *Eucalyptus globulus* wood feedstock. The modification of lignin in the pretreated lignocellulosic material was analyzed by pyrolysis coupled to gas chromatography/mass spectrometry (Py-GC/MS) and two-dimensional nuclear magnetic resonance (2D-NMR) spectroscopy of the whole sample at the gel state [30,31]. Additionally, lignin was isolated from the pretreated samples and further characterized by 2D-NMR. In addition to lignin modification and removal, the effect of the laccase-mediator on the saccharification yield from the pretreated eucalypt feedstock was assessed.

2. Results

2.1. Delignification of eucalypt wood by laccase with and without methyl syringate

Two doses of *M. thermophila* laccase (10 U g^{-1} and 50 U g^{-1}) [24] and methyl syringate (1% and 3%) were tested in the enzymatic pretreatment of eucalypt wood feedstock. This consisted of a sequence of four laccase-mediator treatments, each followed by an alkaline extraction step. The lignin contents of eucalypt samples after the whole laccase-mediator sequence were determined (as Klason lignin) and compared with their respective controls (**Table 1**). The amount of lignin decreased considerably after the enzymatic sequence, concomitantly with increasing laccase doses. The decreases were about 37% and 47% of the initial lignin content when using laccase doses of 10 U g^{-1} and 50 U g^{-1} in combination with 1% and 3% methyl syringate, respectively. The treatments with laccase alone (without mediator) decreased the lignin content about 12% and 20% when using laccase doses of 10 U g^{-1} and 50 U g^{-1}, respectively.

Table 1. Lignin content and monosaccharides release **(%** of sample weight**)** by cellulase hydrolysis of eucalypt samples.

Eucalypt samples	Lignin (%)	Glucose (%)	Xylose (%)
Initial eucalypt wood	22.3 ±0.3	39.5 ±1.1	6.7 ±0.1
Control	21.1 ±1.0	43.7 ±0.2	7.5 ±0.1
Laccase (10 U g^{-1}) - MeS (1%)	13.3 ±0.1	54.8 ±1.0	9.2 ±0.2
Laccase (50 U g^{-1}) - MeS (3%)	11.2 ±0.3	55.7 ±0.4	9.1 ±0.1
Laccase (10 U g^{-1})	18.5 ±0.4	46.3 ±0.8	7.6 ±0.1
Laccase (50 U g^{-1})	16.8 ±0.3	47.8 ±1.2	8.1 ±0.2

Lignin content (as Klason lignin) and monosaccharides from cellulase hydrolysis of eucalypt samples treated with *M. thermophila* laccase (10 U·g^{-1} and 50 U·g^{-1}) and methyl syringate (MeS) mediator (1% and 3%) in a sequence including four enzymatic treatments (and four alkaline peroxide extractions) compared with a control without enzyme, a treatment with laccase alone, and the initial eucalypt wood. Means ± SD (from triplicates).

2.2. Enzymatic hydrolysis of pretreated eucalypt wood

The wood samples treated with laccase (10 U g^{-1} and 50 U g^{-1}), alone and in the presence of methyl syringate (1% and 3%, respectively), as well as the corresponding controls (and the initial untreated wood) were hydrolyzed (72 h) using a cellulase and β-glucosidase cocktail [24], and

the main monosaccharides released (glucose and xylose) were analyzed by GC. When low cellulase (2 filter-paper units [FPU] g^{-1}) and β-glucosidase (100 nkat g^{-1}) doses were used, increases in glucose yields up to 39% and 41% (with respect to the initial eucalypt wood sample) were attained in the samples pretreated with 10 U g^{-1} and 50 U g^{-1} of laccase, in combination with 1% and 3% mediator, respectively (**Table 1**). In the samples pretreated with 10 U g^{-1} and 50 U g^{-1} of laccase alone (without mediator), increases in glucose release of 17% and 21%, respectively, were produced. The effect of oxygen and alkaline extraction steps in the control sample were responsible for the increase of 11% in glucose yield with respect to the initial eucalypt sample. An improvement on xylose release of about 37% was obtained after the laccase-mediator treatment of eucalypt wood (with respect to the initial eucalypt wood sample). This improvement was similar with the two different doses of laccase-mediator used. However, in the pretreatment with laccase alone, different increases in xylose yields (13% and 21%) were obtained with the two laccase doses (10 U g^{-1} and 50 U g^{-1}, respectively). The effect of oxygen and alkaline extraction on xylose yield (control sample with respect to the initial one) represented an increase of 12%.

2.3. Pyrolysis coupled to gas chromatography/mass spectrometry of pretreated eucalypt wood

Modification of the eucalypt lignin by the enzymatic pretreatment was studied by Py-GC/MS. This degradative technique allows for *in situ* analysis of lignin by chromatographic separation and mass-spectrometric identification of the compounds released after the pyrolytic breakdown of whole wood samples (**Table 2**). The main lignin-derived compounds (lignin markers) released were guaiacol, 4-methylguaiacol, 4-ethylguaiacol, 4-vinylguaiacol, syringol, 4-methylsyringol, *trans*-isoeugenol, 4-ethylsyringol, 4-vinylsyringol, 4-allylsyringol, *cis*-4-propenylsyringol, syringaldehyde, *trans*-4-propenylsyringol and *trans*-sinapaldehyde.

The results of the Py-GC/MS analyses of the control wood indicated that the eucalypt lignin was rich in S-units, with a syringyl-to-guaiacyl (S/G) ratio of 4.0 for the control sample. Interestingly, the laccase-mediator treatment caused a decrease in the G-lignin units with respect to the S-lignin ones, resulting in an increase of the S/G ratio to 4.9. The decrease of phenylpropane type compounds was also noticeable, as shown by the ratio between reduced-chain (C_6-C_{0-2}) and full-chain (C_6-C_3) lignin markers, which increased from 2.0 (control wood) to 3.2 in the laccase-mediator-treated sample, revealing cleavage of the lignin unit side-chains by the enzymatic pretreatment. Both the side-chain reduction and the S/G ratio increase tendencies were also observed in the eucalypt wood pretreated with laccase alone, although the changes produced were

much more moderate than those obtained in the presence of methyl syringate.

Table 2. Relative molar abundances of lignin markers from Py-GC/MS of eucalypt wood treated with laccase-mediator, laccase alone and control.

Compound	Control	Laccase only	Laccase and methyl syringate
Guaiacol (G)	4.4	4.8	6.4
4-methylguaiacol (G-CH3)	2.6	2.0	1.2
4-ethylguaiacol (G-CH2-CH3)	1.0	1.2	1.2
4-vinylguaiacol (G-CH=CH2)	4.0	3.5	2.9
Eugenol (G-CH2-CH=CH2)	0.9	0.8	0.5
Syringol (S)	19.2	21.7	32.0
cis-isoeugenol (G-CH=CH-CH3)	0.7	0.5	0.3
trans-isoeugenol (G-CH=CH-CH3)	4.0	3.6	2.0
4-methylsyringol (S-CH3)	7.5	7.2	5.0
Vanillin (G-CHO)	1.3	1.0	0.7
4-ethylsyringol (S-CH2-CH3)	3.5	3.8	5.3
Acetovanillone [G-CO-CH3]	0.7	0.9	1.1
4-vinylsyringol (S-CH=CH2)	13.3	12.4	12.3
Guaiacylacetone (G-CH2-CO-CH3)	0.5	0.5	0.5
4-allylsyringol (S-CH2-CH=CH2) + 4-propylsyringol (S-CH2-CH2-CH3)	4.3	4.1	3.6
cis-propenylsyringol (S-CH=CH-CH3)	2.6	2.3	1.8
trans-propenylsyringol (S-CH=CH-CH3)	14.4	12.8	10.2
Syringaldehyde (S-CHO)	5.8	4.5	2.5
Homosyringaldehyde (S-CH2-CHO)	0.0	1.1	0.9
Acetosyringone (S-CO-CH3)	3.6	5.0	4.9
Syringylacetone (S-CH2-CO-CH3)	2.2	2.2	2.3
Propiosyringone (S-CO-CH2-CH3)	0.7	0.8	0.6
Dihydrosinapyl alcohol (S-CH2-CH2-CH2OH)	0.7	0.8	0.5
trans-sinapyl alcohol (S-CH=CH-CH2OH)	0.8	0.2	0.3
trans-sinapaldehyde (S-CH=CH-CHO)	1.0	2.3	1.0
C_6-C_{0-2}/C_6-C_3 ratio	2.0	2.2	3.2
Syringyl-to-guaiacyl ratio	4.0	4.3	4.9

Main lignin-derived compounds (lignin markers) from Py-GC/MS of eucalypt wood treated with *M. thermophila* laccase (50 U g^{-1}) and methyl syringate (3%) in a sequence including four enzymatic treatments and four alkaline peroxide extractions compared with a control without enzyme, and a treatment with laccase alone. Methyl syringate was also recovered among the Py-GC/MS products from the laccase and methyl syringate sample (amounting to 8% of the listed lignin-derived markers). The ratio between lignin markers with reduced side chains (C_6-C_{0-2}) and phenylpropane (C_6-C_3) markers, as well as the syringyl-to-guaiacyl ratio, are also indicated.

2.4. Two-dimensional nuclear magnetic resonance of pretreated eucalypt wood

The modification of lignin structure after the whole enzymatic pretreatments of eucalypt wood was also studied by 2D-NMR. With this purpose, the wood samples were swelled in deuterated dimethylsufoxide (DMSO-d_6) forming a gel, and analyzed by heteronuclear single quantum correlation (HSQC) NMR. **Figure 1** shows the HSQC spectra of the wood samples before (initial wood), after laccase (alone) and after laccase-mediator treatments with the higher laccase (50 $U \cdot g^{-1}$) and mediator (3%) doses. The control treatment, without enzyme and mediator, was also analyzed. The aliphatic oxygenated region of the spectra shows methoxyl, lignin side-chain and carbohydrate cross-signals, and the aromatic region, include the signals of S- and G-lignin units. The main lignin structures identified are shown in **Figure 2**, and the different lignin signals assigned on the spectra are listed in **Table 3**. **Table 4** shows the composition of lignin, in terms of S and G units, and the relative abundance of the main inter-unit linkages in the different samples, which were estimated from the signal volume integrals.

The aliphatic-oxygenated region of the HSQC spectrum of the initial eucalypt wood (**Figure 1A**, top-right) shows signals of lignin and carbohydrates, the latter mainly corresponding to xylan (X) and acetylated xylan (X') units, since crystalline cellulose is nearly 'silent' in lignocellulose gel spectra under solution NMR conditions. In this region, signals of side-chains in β-*O*-4' alkyl-aryl ether lignin substructures (A), including C_γ-H_γ, C_β-H_β and C_α-H_α correlations (A_γ, A_β and A_α, respectively) were observed. The A_γ signal overlapped with related signals in lignin and other lignocellulose constituents. The C_β-H_β correlations gave two different signals corresponding to β-*O*-4' substructures where the second unit was an S unit or a G unit ($A_{\beta(S)}$ and $A_{\beta(G)}$), the latter with lower intensity, in agreement with lignin composition described below. Other less prominent signals for resinol (β–β') substructures (C) were also observed in the spectrum, with their C_α–H_α, C_β–H_β and the double C_γ–H_γ correlations (C_α, C_β and C_γ). The main signals in the aromatic region of the HSQC spectrum (**Figure 1A**, bottom-left) corresponded to the benzenic rings of the S and G lignin units. The S-lignin units showed a prominent signal for the $C_{2,6}$-$H_{2,6}$ correlation ($S_{2,6}$), whereas the G-lignin units showed different correlations for C_2-H_2 (G_2), C_5-H_5 (G_5) and C_6-H_6 (G_6). Signals corresponding to $C_{2,6}$-$H_{2,6}$ correlations in C_α-oxidized S-lignin units ($S'_{2,6}$) were also observed although in low quantities. From the integrals of the above signals, an S/G ratio around 3.6, and a large predominance of β-*O*-4' ether linkages together with some resinols, was estimated for lignin in *E. globulus* wood (**Table 4**).

The HSQC spectrum of the eucalypt control sample at the end of the whole sequence (**Figure 1B**) showed some differences compared to the initial eucalypt. The most remarkable was the disappearance of the

signals of the acetylated xylan units (X'). This may have been caused by the conditions of the control treatment (oxygen addition and alkaline peroxide extractions) that was performed under the same conditions as the enzyme-pretreated samples except for the addition of enzyme and mediator. Concerning lignin side chains, the spectrum of the control sample also revealed some decrease in the amount of β-*O*-4' alkyl-aryl ethers (about 21%) and resinol (about 60%) substructures per 100 phenylpropane units, with respect to the initial wood (**Table 4**). Less intense signals of G and $S'_{2,6}$ units than in the initial eucalypt wood were also observed in the aromatic region.

The HSQC spectrum of the eucalypt sample treated with laccase-mediator at the end of the whole sequence (**Figure 1D**) showed important differences compared to the control. The signals of side-chains in β-*O*-4' lignin substructures (A_α and $A_{\beta(S)}$) decreased considerably with respect to the carbohydrate and S-lignin signals. The G lignin signals completely disappeared with the laccase-mediator treatment, whereas the S units were C_α-oxidized (and in a significant extent remained as such), as revealed by the increase in the $S'_{2,6}$ signal. The results obtained showed a C_α-oxidation mechanism for lignin removal by laccase in the presence of methyl syringate, and revealed that about half of the residual lignin in the laccase-mediator-treated wood corresponded to the C_α-oxidized S units, as shown in **Table 4** where the contribution of methyl syringate to the 106/7.3 ppm signal was subtracted. Finally, the low intensity of the aromatic and aliphatic-oxygenated lignin signals in the HSQC spectrum of the laccase-mediator-treated sample, compared to the carbohydrate signals, was in agreement with the reduced Klason lignin content (**Table 1**).

Interestingly, lignin modification and removal was also shown by the NMR spectra of the eucalypt feedstock treated with laccase alone (**Figure 1C**), with a relative decrease of the lignin signals compared to the carbohydrate signals, although not as evident as that observed after the laccase-mediator treatment. Among them, the signals of side-chains in β-*O*-4' lignin substructures (A_α and $A_{\beta(S)}$) and especially the G lignin signals, decreased considerably with respect to the control sample (**Table 4**), although the changes were less intense than those found in the sample treated with laccase and methyl syringate. Likewise, the C_α-oxidation of S units was much less pronounced than found in the presence of methyl syringate.

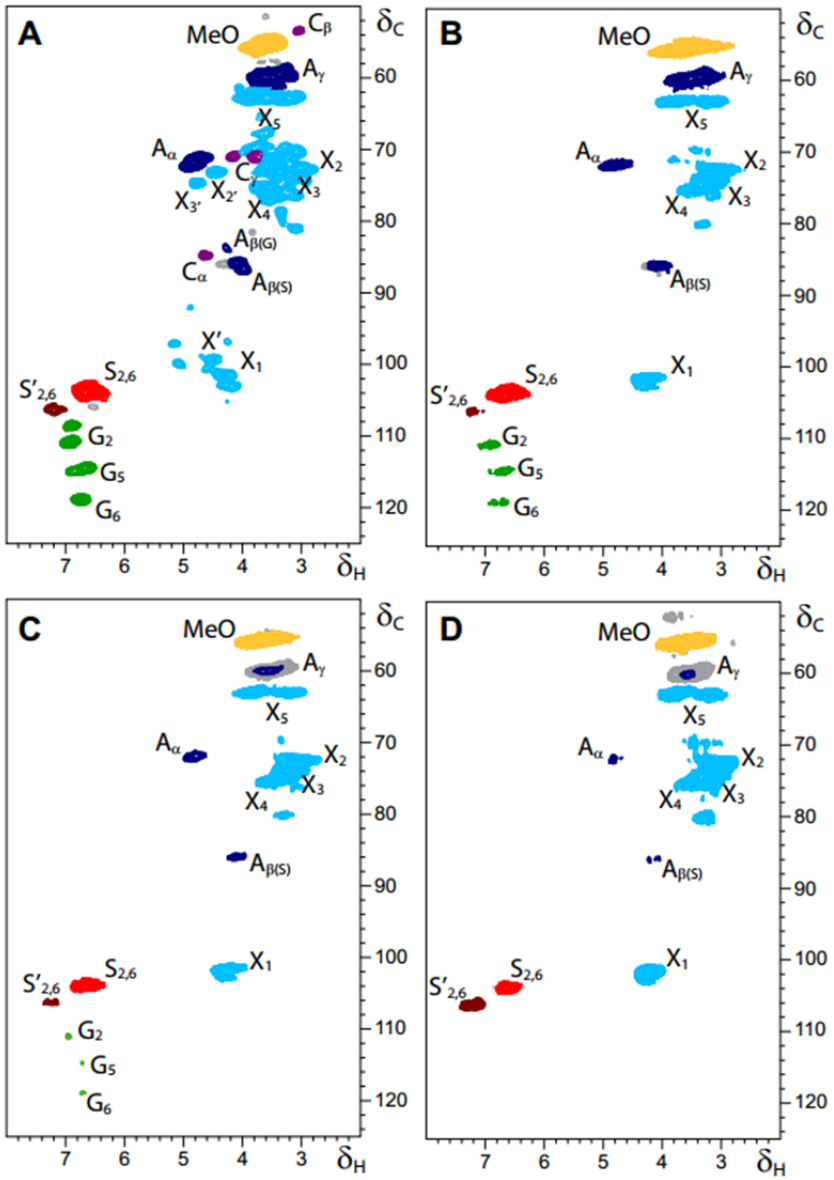

Figure 1. Heteronuclear single quantum correlation nuclear magnetic resonance spectra of whole eucalypt samples swollen in dimethylsulfoxide-d_6. **(A)** Initial sample, **(B)** control without enzyme, **(C)** sample treated with laccase alone (50 U g^{-1}) and **(D)** sample treated with laccase (50 U g^{-1}) and methyl syringate (3%). See **Table 3** for lignin signal assignment, **Figure 2** for the main lignin structures identified and **Table 4** for quantification of these lignin structures. Correlation signals from normal (X_1-X_5) and acetylated xylan (X'_1-X'_3) are also indicated. The 52/3.8 ppm signal corresponds to some methyl syringate incorporated onto the lignin (see **Figure 3**). The enzymatic pretreatment included four laccase-mediator treatments, each followed by an alkaline extraction step.

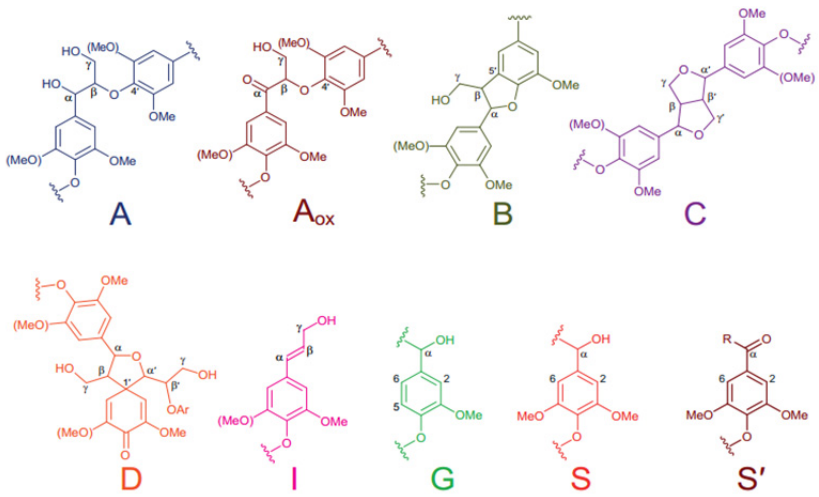

Figure 2. Main lignin structures identified in the eucalypt samples analyzed by heteronuclear single quantum correlation nuclear magnetic resonance (**Figures 1** and **3**). **A** = β-*O*-4' lignin substructures (including a second S or G unit); Aox = C_{α}-oxidized β-*O*-4' lignin substructures; **B** = phenylcoumarans; **C** = resinols; **D** = spirodienones; **I** = cinnamyl alcohol end-groups; **G** = guaiacyl units; **S** = syringyl units; and **S'** = C_{α}-oxidized S units (R can be a hydroxyl in carboxylic acids or a lignin side-chain in ketones).

2.5. Two-dimensional nuclear magnetic resonance of lignin isolated from pretreated eucalypt wood

To gain further insight into the modification of lignin structure with the laccase-mediator treatment, cellulolytic enzyme lignin (CEL) was isolated from the pretreated eucalypt samples, and analyzed by 2D-NMR (**Figure 3**). The lignin structures identified, some of them not detected in the wood spectra, are shown in **Figure 2**, and the corresponding signals are listed in **Table 2**. **Table 5** shows the lignin composition, and percentages of inter-unit linkages (and end-groups) in the different CEL samples, estimated from the signal volume integrals as described for the wood spectra.

The HSQC spectrum of the CEL preparation isolated from the initial eucalypt sample (**Figure 3A**) showed the same side-chain signals observed in the spectrum of the whole eucalypt sample (**Figure 1A**). These corresponded to β-*O*-4' alkyl-aryl ether (A) and resinol (C) correlations, although were better resolved and with higher intensity, and several new signals that could not be observed in the wood spectra. The latter included: spirodienone (β-1'/α-O-α') substructures (D) with their C_{α}-H_{α}, $C_{\alpha'}$-$H_{\alpha'}$, C_{β}-H_{β} and $C_{\beta'}$-$H_{\beta'}$ correlations (D_{α}, $D_{\alpha'}$, D_{β}, $D_{\beta'}$); small signals

corresponding to phenylcoumaran (β-5') substructures (B) with their C_α-H_α, C_β-H_β and C_γ-H_γ correlations (B_α, B_β and B_γ; the two latter correlations overlapping with other signals); a signal of cinnamyl alcohol end-groups (I) with its C_γ-H_γ correlation; and signals of C_β-H_β correlations in C_α-oxidized β-*O*-4' alkyl-aryl ether substructures ($A_{ox\beta}$) (**Figure 2** and **Table 5**). The main signals in the aromatic region of the spectrum of the initial eucalypt CEL sample corresponded to the benzenic rings of the S and G lignin units as shown for the whole wood spectrum. Signals from C_α-oxidized S-lignin units ($S'_{2,6}$) were also observed. Some new signals not observed in the wood spectrum appeared in this region corresponding to the above-mentioned spirodienone substructure (D) with C_2-H_2 and C_6-H_6 correlations (D_2 and D_6).

Table 3. Assignments of lignin main ^{13}C-^{1}H correlation signals in the heteronuclear single quantum correlation spectra of eucalypt wood and lignins.

Label	δ_C/δ_H (ppm)	Assignment
B_β	53.1/3.45	C_β−H_β in phenylcoumaran substructures (**B**)
C_β	53.3/3.05	C_β−H_β in β−β' resinol substructures (**C**)
MeO	55.6/3.72	C−H in methoxyls
A_γ	59.4 /3.38 and 3.70	C_γ−H_γ in β-*O*-4' structures (**A**)
D_β	59.7/2.73	C_β−H_β in spirodienone substructures (**D**)
I_γ	61.3/4.08	C_γ−H_γ in cinnamyl alcohol end-groups (**I**)
B_γ	62.6/3.67	C_γ−H_γ in phenylcoumaran substructures (**B**)
A_α	71.8/4.85	C_α−H_α in β-*O*-4' structures (**A**)
C_γ	71.0/ 3.81 and 4.18	C_γ−H_γ in β−β' resinol substructures (**C**)
$D_{\beta'}$	79.2/4.10	$C_{\beta'}$−$H_{\beta'}$ in spirodienone substructures (**D**)
D_α	81.0/5.08	C_α−H_α in spirodienone substructures (**D**)
$A_{ox\beta}$	83.0/5.20	C_β−H_β in α-oxidized β-O-4′ substructures (**A_{ox}**)
$A_{\beta(G)}$	83.6/4.28	C_β−H_β in β-*O*-4' structures (**A**) linked to a G-unit
$D_{\alpha'}$	83.7/4.68	$C_{\alpha'}$−$H_{\alpha'}$ in spirodienone substructures (**D**)
C_α	84.7/4.64	C_α−H_α in β−β′ resinol substructures (**C**)
$A_{\beta(S)}$	85.7/4.10	C_β−H_β in β-*O*-4' structures (A) linked to a S-unit
B_α	86.4/5.43	C_α−H_α in phenylcoumaran substructures (**B**)
$S_{2,6}$	103.9/6.69	C_2−H_2 and C_6−H_6 in syringyl units (**S**)
$S'_{2,6}$	106.1/7.18 and 7.31	C_2−H_2 and C_6−H_6 in α-oxidized syringyl units (**S'**)
G_2	110.8/6.96	C_2−H_2 in guaiacyl units (**G**)
D_2	113.3/6.25	C_2−H_2 in spirodienone substructures (**D**)
G_5	114.3/6.69, 114.9/6.94	C_5−H_5 in guaiacyl units (**G**)
G_6	118.8/6.78	C_6−H_6 in guaiacyl units (**G**)
D_6	118.7/6.06	C_6−H_6 in spirodienone substructures (**D**)

From heteronuclear single quantum correlation spectra in **Figures 1** and **3**. See **Figure 2** for chemical structures indicated by letters in bold.

In the case of CEL preparations, the HSQC spectrum of the control sample (**Figure 3B**) was very similar to that of the initial material described above, although with less intense carbohydrate signals (due to the lower polysaccharide content of the control wood sample) and a slightly higher intensity of some lignin signals (**Table 5**).

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Table 4. Lignin units and inter-unit linkages from the heteronuclear single quantum correlation spectra of treated eucalypt wood and controls.

	Wood	Control	Laccase only	Laccase and methyl syringate
Lignin units				
Syringyl (**S**) (% total)	78	79	86	100
Guaiacyl (**G**) (% total)	22	21	14	0
C_α-oxidized S units (**S'**) (% S)	13	11	16	47
S/G ratio	3.6	3.8	6.3	-
Lignin inter-unit linkages (% *S+G*)				
β-*O*-4' Alkyl-aryl ethers (**A**)	61 (86)	48 (92)	42 (100)	23 (100)
Phenylcoumarans (**B**)	0	0	0	0
Resinols (**C**)	10 (14)	4 (8)	0	0
Spirodienones (**D**)	0	0	0	0
Total	71 (100)	52 (100)	42 (100)	23 (100)

The lignin composition (S and G units), the amount of C_α-oxidized S units (with respect to total S units), the S/G ratio, and the abundance of side chains forming different inter-unit linkages (A-D) per 100 phenylpropane units were determined from the heteronuclear single quantum spectra of eucalypt wood treated with laccase (50 U g^{-1}) and 3% methyl syringate and laccase alone, compared with a control without enzyme and the initial wood. The percentage of side chains forming each linkage type (or end-group) are also indicated in parentheses.

The HSQC spectra of isolated lignins from the eucalypt samples after enzymatic pretreatment are shown in **Figure 3C**,**D**. The main differences in lignin G units and inter-unit linkages, compared with the previous samples, are shown in **Table 5**. Concerning lignin composition, the most noticeable effect of the enzymatic treatments of the residual lignin remaining in wood was the significant reduction in G units produced by the laccase-mediator treatment (**Figure 3D**), resulting in an increase of the S/G ratio from 4.3 to 14.1. Additionally, a strong increase in C_α-oxidized S units was produced, as shown in **Table 5** where the contribution of methyl syringate to the lignin $S'_{2,6}$ signal at 106/7.3 ppm was deduced. The increase of the $S'_{2,6}$ aromatic signal was accompanied by an increase in the C_β-H_β correlations signal from β-*O*-4' ether linked

C_α-oxidized side chains ($A_{ox\beta}$). Moreover, a significant decrease in β-*O*-4' alkyl-aryl ether (A) and resinol substructures (C) per 100 phenylpropane units was the main effect observed in the side-chain region of the HSQC spectra of the lignin isolated from the wood treated with laccase and methyl syringate with the laccase-mediator treatment, together with a decrease in the less intense signals of phenylcoumarans, spirodienones and cinnamyl end-groups.

The 2D-NMR analysis of lignin isolated from eucalypt samples pretreated with laccase alone (**Figure 3C**) also revealed some differences with respect to the control. Interestingly, the most remarkable effect was the increase of oxidized lignin structures evidenced by both aromatic ($S'_{2,6}$) and aliphatic side-chains ($A_{ox\beta}$) signals (**Table 5**), revealing that laccase alone attacks lignin by a mechanism similar to that of the laccase-mediator system

Table 5. Lignin units and inter-unit linkages (and end-groups) from the heteronuclear single quantum correlation spectra of cellulolytic enzyme lignin preparations from treated wood and controls.

Lignin structure	Wood	Control	Laccase only	Laccase and methyl syringate
Lignin units				
Syringyl (**S**) (% total)	77	81	83	93
Guaiacyl (**G**) (% total)	23	19	17	7
C_α-oxidized S units (**S'**) (% S)	6	10	18	35
S/G ratio	3.4	4.3	4.9	14.1
Inter-unit linkages and end-groups				
β-*O*-4' Alkyl-aryl ethers (**A**) (% S+G)	58 (82)	61 (84)	56 (85)	49 (92)
Phenylcoumarans (**B**) (% S+G)	2 (3)	1 (1)	1 (2)	0
Resinols (**C**) (% S+G)	9 (13)	8 (11)	6 (9)	2 (4)
Spirodienones (**D**) (% S+G)	2 (3)	2 (3)	2 (3)	1 (2)
Cinnamyl end-groups (**I**) (% S+G)	1 (1)	1 (1)	1 (1)	0 (1)
Total (% S+G)	71 (100)	73 (100)	66 (100)	53 (100)
C_α-oxidized β-*O*-4' ethers ($\mathbf{A_{ox}}$) (% A)	2	2	5	14

The lignin composition (S and G units), the amount of C_α-oxidized S units (with respect to total S units), the S/G ratio, the abundance of side chains forming different inter-unit linkages (A-D) and cinnamyl end-groups (I) per 100 phenylpropane units, and the relative abundance of C_α-oxidized β-O-4' ethers (with respect to total β-O-4' ethers) were determined from the heteronuclear single quantum correlation spectra of cellulolytic enzyme lignin and laccase alone, compared with a control without enzyme and the initial wood. The percentage of side chains forming each linkage type (or end-group) are also indicated (parentheses).

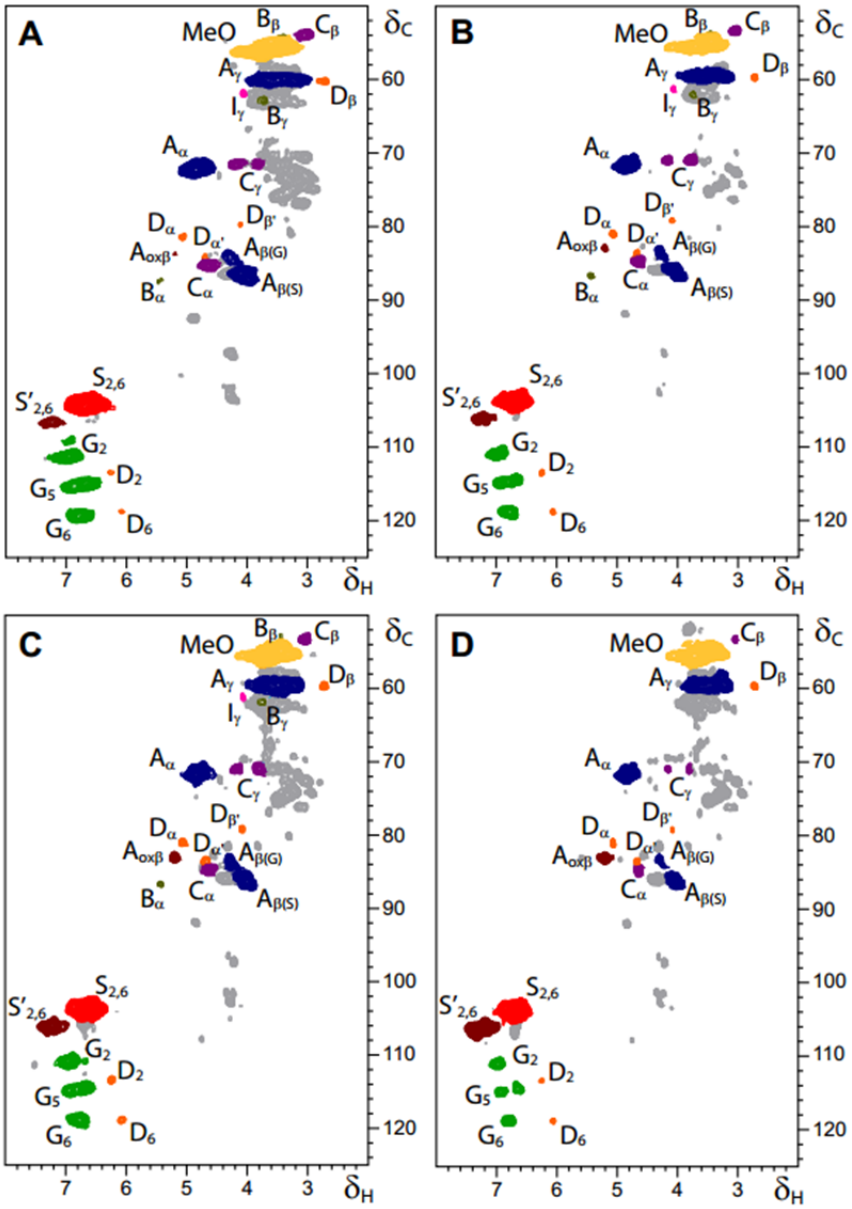

Figure 3. Heteronuclear single quantum correlation nuclear magnetic resonance spectra of cellulolytic enzyme lignins isolated from eucalypt wood samples**. (A)** Initial eucalypt sample, **(B)** control without enzyme, **(C)** sample treated with laccase alone (50 U g^{-1}) and **(D)** sample treated with laccase (50 U g^{-1}) and methyl syringate (3%). See **Table 3** for lignin signal assignment, **Figure 2** for the main lignin structures identified, and **Table 5** for quantification of these lignin structures identified. The 52/3.8 ppm signal corresponds to some methyl syringate incorporated onto the lignin. The enzymatic pretreatment included four laccase-mediator treatments, each followed by an alkaline extraction step.

3. Discussion

This work shows the potential of an oxidative enzymatic pretreatment to delignify a hardwood feedstock (*E. globulus* wood) and improve its enzymatic saccharification. Eucalypt is a rapidly growing and high biomass-producing tree used as a raw material for paper pulp manufacturing in several countries, including southwest Europe, Brazil and South Africa. Among the different eucalypt species, wood from *E. globulus* is the best raw material for kraft pulp manufacturing due to the high pulp yield [32]. Additionally, the lignin of *E. globulus* is enriched in S units with β-*O*-4' linkages predominating [32-34], which implies principally linear chains with less cross-linking than G-rich lignin because of the methoxylated and, therefore, blocked C-5 position in the S units. With all these characteristics, *E. globulus* wood has great potential as a lignocellulosic feedstock for the production of second-generation wood-based bioethanol [35].

3.1. Delignification and improved saccharification by laccase (and methyl syringate)

Because carbohydrates in intact wood are not amenable to enzymatic hydrolysis, the use of some type of pretreatment is needed for the production of bioethanol [11,12]. To overcome the recalcitrance of lignocellulosic biomass associated with lignin, a delignification strategy based on the use of the laccase from the ascomycete *M. thermophila* and the phenolic mediator methyl syringate is reported here. Lignin removal of up to 50% from *E. globulus* wood was attained using this laccase-mediator pretreatment (with 50 U g^{-1} of laccase and 3% of methyl syringate) followed by an alkaline peroxide extraction in a multistage sequence. The enzymatic pretreatments using laccase alone (without mediator) removed 20% of lignin from eucalypt samples (compared with 5% delignification in the control sample). This result suggests the involvement of natural phenolic structures mediating the enzymatic oxidation.

No significant decrease in the lignin content had been shown to date after laccase (alone) treatment of lignocellulosic feedstocks, such as steam-pretreated giant reed and Northern spruce [36]. Likewise, no substantial variation in lignin content and composition was reported after laccase-mediator treatment of steam-exploded eucalypt wood with *M. thermophila* laccase (Novozym 51003 from Novozymes) [37]. This is most probably due to the different mediator, HBT, which is scarcely oxidized by *M. thermophila* laccase [38], and treatment conditions, although the modified lignin structure after steam explosion could also have some influence. *M. thermophila* and other ascomycete laccases have lower redox potential than basidiomycete laccases [39]; however, this is not a

problem when an easily oxidized phenolic mediator is used, as shown for paper pulp bleaching [29]. In return, ascomycete enzymes are more easily over-expressed in industrial hosts as recombinant proteins, enabling their commercialization for industrial applications (as in the case of *M. thermophila* laccase produced by Novozymes).

Enzymatic removal of lignin from ground eucalypt wood had been recently reported by Gutiérrez *et al*. [24] using the high redox-potential laccase from *Trametes villosa* and HBT as mediator. However, the cost, safety and environmental profile of this synthetic mediator make its implementation at an industrial scale difficult. To overcome these limitations, several lignin-derived phenols selected as natural laccase mediators [40] have been investigated for paper pulp biobleaching [27-29]. Our results showing methyl syringate helping laccase to delignify eucalypt wood are in agreement with laccase oxidation of non-phenolic model compounds in the presence of this and related phenolic mediators [41]. The present paper shows the potential of using a commercial laccase and a natural phenol as a mediator for delignifying a woody feedstock. The enzyme used is the thermostable laccase from the ascomycete *M. thermophila*, which has been cloned, expressed in *Aspergillus oryzae*, biochemically characterized, improved for different applications, and commercialized [39,42]. The mediator used, methyl syringate, is a natural product and shows promising results as a laccase mediator due to a suitable redox potential [43]. The above combination (of commercial enzyme and low-cost mediator) facilitates industrial feasibility of the laccase-mediator pretreatment.

As expected from the decrease of (Klason) lignin content, the pretreatment of eucalypt wood with the *M. thermophila* laccase and methyl syringate improved the saccharification yield similarly (about 40%) with the two different doses of laccase (10 U g^{-1} and 50 U g^{-1}) and mediator (1% and 3%, respectively) used. An improvement on cellulose hydrolysis of ensiled corn stover was attained by the use of laccase from *Trametes versicolor* and HBT using higher doses of laccase (4,000 U g^{-1}) and mediator (5%) and cellulolytic enzymes (15 FPU g^{-1} cellulase, and 1,000 nkat g^{-1} β-glucosidase) [25]. Likewise, an improvement in cellulose hydrolysis was reported when treating steam-pretreated softwood with *Trametes hirsuta* laccase and the mediator *N*-hydroxy-*N*-phenylacetamide [23]. The relatively low doses of cellulases used in the present work (2 FPU g^{1} Celluclast 1.5L and 100 nkat g^{-1} of β-glucosidase) to obtain a glucose yield of 55% are noteworthy. As a significant decrease in the cost of cellulases is necessary for the economic conversion of lignocellulose to ethanol [44], high hydrolysis yields at low dosage of cellulases are highly desirable.

3.2. Structural modification of lignin by laccase (and methyl syringate)

During wood delignification by laccase and methyl syringate, an important fraction of the lignin is depolymerized and released from the sample causing the 50% reduction of Klason lignin. However, 2D-NMR and other analyses of the whole wood and its isolated CEL revealed that the residual lignin remaining in wood is also modified during the enzymatic pretreatment.

The general structure of the eucalypt lignin, in terms of aromatic units and inter-unit linkages, agrees with that reported in previous studies [30,32-34]. The most important modification of the residual lignin during wood pretreatment with laccase and methyl syringate was the strong increase in C_α-oxidized (main) S units, revealed by 2D-NMR analyses of the whole wood and the CEL preparations. Another noticeable effect shown by the 2D-NMR analyses, and also by Py-GC/MS, was the relative reduction in G lignin units produced by the laccase-mediator treatment, resulting in a strong increase of the S/G ratio (lignin composition by 2D-NMR also includes condensed structures that are recalcitrant towards pyrolytic breakdown). The decrease in lignin G units, which occurred to a greater extent than that of the S lignin units, has previously been observed in the pretreatment of eucalypt wood with *T. villosa* laccase and HBT [24]. Moreover, a significant decrease in the number of side-chains involved in the different lignin substructures (per 100 phenylpropane units) was observed after the laccase treatment in the presence of methyl syringate, in agreement with progressive depolymerization.

Generation of oxidized lignin structures is congruent with the nature of the lignin biodegradation process, which has been described as an 'enzymatic combustion' where fungal oxidoreductases play a central role [45]. It is generally accepted that lignin degradation by white rot fungi and their ligninolytic peroxidases starts by aromatic ring oxidation, but quickly leads to side-chain C_α-C_β cleavage, causing depolymerization [46]. The same mechanism has been suggested for some laccase reactions mediated by synthetic compounds, for example 2,2-azinobis(3-ethylbenzothiazoline-6-sulfonate), but the action of laccase-HBT on non-phenolic lignin models is predominantly produced by hydrogen-atom abstraction from the C_α position, followed by alkyl-aryl ether breakdown [47,48].

The above attack mechanism would result in the increased amount of C_α-oxidized S lignin units found in eucalypt wood pretreated with *T. villosa* laccase and HBT [24]. The presence of oxidized S lignin units was also observed in eucalypt pulp residual lignin after laccase-HBT treatment, including both C_α ketones and carboxylic acids [49]. Additionally, lignin side-chain oxidation by laccase-HBT has been reported after Py-GC/MS and thermochemolysis of laccase-treated eucalypt wood and corn stover,

respectively [25,50]. Wood lignin modification by laccase in the presence of methyl syringate also yielded a structural modification pattern characterized by extensive C_α-oxidation (as shown by 2D-NMR), suggesting that the attack mechanism by laccase in the presence of methyl syringate is the same reported by laccase-HBT. This agrees with results from model compounds showing that laccase in the presence of phenolic mediators oxidizes non-phenolic aromatic compounds *via* a hydrogen abstraction mechanism [51].

4. Conclusions

Eucalypt feedstock can be delignified by a high-yield expressed (recombinant) laccase when applied in a sequence consisting of successive enzymatic and alkaline extraction stages, directly on the ground lignocellulosic material (that is, without a previous deconstruction pretreatment). Lignin removal reached 50% when methyl syringate, a natural and potentially cheap mediator, was applied together with the enzyme. The pretreated eucalypt feedstock was hydrolyzed with higher efficiency than the untreated material, releasing higher yields of glucose and xylose using relatively low doses of cellulases. Preferential removal of lignin G units, in comparison to S units, and breakdown of main inter-unit linkages was suggested by Py-GC/MS, and confirmed by 2D-NMR. The 2D-NMR spectra of whole wood (at the gel stage) also showed: the selective action of laccase-mediator on the lignin moiety, while the polysaccharide signals remained unchanged with respect to the controls; and the extensive presence of oxidized S units in the residual lignin remaining in pretreated wood. These and other changes in lignin structure were analyzed in depth by 2D-NMR of isolated lignins. The above results provide evidence for a C_α-oxidation mechanism of lignin degradation even on treatment with laccase alone.

5. Methods

5.1. Wood, enzyme and mediator

Eucalypt (*E. globulus*) wood chips from ENCE (Pontevedra, Spain) were air-dried and ground in an IKA MF10 cutting mill to pass through a 100-mesh screen, and then finely milled using a Retsch PM100 planetary mill (Retsch, Haan, Germany) at 400 rev min^{-1} (with 5 min breaks after every 5 min of milling) using a 500 mL agate jar and agate ball bearings (20×20 mm). The total ball-milling time for the samples was 5 h.

A commercial (recombinant) fungal laccase from the ascomycete *M. thermophila*, provided by Novozymes (Bagsvaerd, Denmark), was used in this study. Its activity was measured as initial velocity during oxidation of 5 mM 2,2'-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) from Roche to its cation radical (ε_{436} 29300 M^{-1} cm^{-1}) in 0.1 M sodium acetate (pH 5) at 24°C. The laccase activity of the enzyme preparation was 945 U/mL. One activity unit (U) was defined as the amount of enzyme transforming 1 µmol of ABTS per min.

Methyl syringate (methyl 4-hydroxy-3,5-dimethoxybenzoate) from Alfa Aesar (Karlsruhe, Germany) was used as the mediator.

5.2. Laccase-mediator treatments

The eucalypt samples were treated with the *M. thermophila* laccase in the presence (and absence) of the mediator methyl syringate. Laccase doses of 10 U g^{-1} and 50 U g^{-1}were assayed, together with 1% and 3% methyl syringate, respectively (doses refer to wood dry weight). The treatments were carried out in 200 mL pressurized bioreactors (Labomat, Mathis, Oberhasli/Zürich, Switzerland) placed in a thermostatic shaker at 170 rev min^{-1} and 50°C, using 10 g (dry weight) samples at 6% consistency (w:w) in 50 mM sodium dihydrogen phosphate (pH 6.5) under O_2 atmosphere (2 bars) for 24 h. After the treatment, the samples were filtered through a Büchner funnel and washed with 1 L of water. In a subsequent step, samples at 6% consistency (w:w) were submitted to a peroxide-reinforced alkaline extraction using 1% (w:w) sodium hydroxide and 3% (w:w) hydrogen peroxide (also with respect to sample dry weight) at 80°C for 90 min, followed by water washing [29]. Cycles of four successive enzyme-extraction treatments were applied. Treatments with laccase (10 U g^{-1} and 50 U g^{-1}) alone (without mediator) and controls without laccase and mediator were also performed (followed in both cases by the corresponding alkaline extractions). A control with mediator alone was not included, taking into account the results from previous studies. Klason lignin content was estimated according to TAPPI Method T222 om-88 [52].

5.3. Saccharification of treated wood

The laccase-pretreated samples were hydrolyzed with a cocktail containing commercial enzymes (from Novozymes, Bagsvaerd) with cellulase (Celluclast 1.5 L; 2 FPU g^{-1}) and β-glucosidase (Novozym 188; 100 nkat g^{-1}) activities, at 1% consistency in 3 mL of 100 mM sodium citrate (pH 5) for 72 h at 45 °C, in a thermostatic shaker at 170 rev min^{-1} (in triplicate experiments).

The different monosaccharides released were determined as alditol acetates [53] by GC. An HP 5890 gas chromatograph (Hewlett-Packard, Hoofddorp, The Netherlands) equipped with a split-splitless injector and a flame ionization detector was used. The injector and detector temperatures were set at 225°C and 250°C, respectively. Samples were injected in the split mode (split ratio 10:1). Helium was used as the carrier gas. The capillary column used was a DB-225 (30 m × 0.25 mm internal diameter, 0.15 μm film thickness) from Agilent J&W (Folsom, CA). The oven was temperature-programmed from 220°C (held for 5 min) to 230°C (held for 5 min) at 2°C min^{-1}. Peaks were quantified by area; glucose, xylose and arabinose were used as standards to elaborate calibration curves. The data from the three replicates were averaged.

5.4. Enzymatic isolation of lignin

The air-dried eucalypt samples were extracted three times with water then three times with 80% ethanol by sonicating in an ultrasonic bath for 30 min each time. CEL preparations were isolated by enzymatically saccharifying polysaccharides as described by Chang *et al*. (1975) [54]. Cellulysin cellulase (Calbiochem), a crude cellulase preparation from *Trichoderma viride* also containing hemicellulase activities, was used. Its activity was ≥10,000 FPU g^{-1} of dry weight. The extractives-free ball-milled material (200 mg) was suspended in 30 mL of 20 mM sodium acetate (pH 5.0) in a 50 mL centrifuge tube, 7.5 mg of Cellulysin was added, and the reaction slurry was incubated at 30°C for 48 h. The solids were pelleted by centrifugation (8,000 rpm, 4°C, 20 min), and the process was repeated with fresh buffer and enzyme, three times. Finally, the residue (CEL) was washed with distillated water, recovered by centrifugation and freeze dried.

5.5. Pyrolysis-gas chromatography/mass spectrometry

Pyrolysis of eucalypt wood samples (approximately 100 μg) was performed with an EGA/PY-3030D micro-furnace pyrolyzer (Frontier Laboratories Ltd., Fukushima, Japan) connected to an Agilent 7820A gas chromatograph using a DB-1701 fused-silica capillary column (60 m x 0.25 mm internal diameter, 0.25 μm film thickness) and an Agilent 5975 mass selective detector (EI at 70 eV). The pyrolysis was performed at 500°C. The oven temperature was programmed from 45°C (4 min) to 280°C (10 min) at 4°C min^{-1}. Helium was the carrier gas (1 mL min^{-1}). The compounds were identified by comparing their mass spectra with those of the Wiley and NIST libraries and reported in the literature [55,56]. Peak molar areas were calculated for the lignin-degradation products, the summed areas were normalized, and the data for three repetitive

analyses were averaged and expressed as percentages. The relative standard deviation for the pyrolysis data was less than 5%.

5.6. Two-dimensional nuclear magnetic resonance spectroscopy

For gel-state NMR experiments, approximately 100 mg of finely divided (ball-milled) extractive-free wood samples were directly transferred into 5 mm NMR tubes, and swelled in 1 mL of DMSO-d_6, forming a gel inside the NMR tube [30,31]. For a more in-depth structural characterization of the lignins, around 30 mg of CEL preparations were dissolved in 0.75 mL of DMSO-d_6.

HSQC 2D-NMR spectra were acquired at 25°C on a Bruker AVANCE III 500 MHz spectrometer (Bruker Biospin, Fallanden, Switzerland) fitted with a cryogenically cooled 5 mm TCI gradient probe with inverse geometry (proton coils closest to the sample). The 2D ^{13}C-^{1}H correlation spectra were carried out using an adiabatic HSQC pulse program (Bruker standard pulse sequence 'hsqcetgpsisp2.2') and the following parameters: spectra were acquired from 10 to 0 ppm (5,000 Hz) in F2 (^{1}H) using 1,000 data points for an acquisition time of 100 ms, an interscan delay of 1 s, and from 200 to 0 ppm (25,168) in F1 (^{13}C) using 256 increments of 32 scan, for a total acquisition time of 2 h 34 min. The $^{1}J_{CH}$ used was 145 Hz. Processing used typical matched Gaussian apodization in ^{1}H and a squared cosine bell in ^{13}C. The central solvent peak was used as an internal reference (δ_C/δ_H 39.5/2.49). The ^{13}C-^{1}H correlation signals from the aromatic region of the spectrum were used to estimate the lignin composition in terms of G, S and oxidized S (S') units, and those of the aliphatic-oxygenated region were used to estimate the inter-unit linkage and end-unit abundances. The quantification was carried out using correction factors based on estimated carbon-proton coupling constants. The S lignin content in the laccase-mediator-treated sample was corrected for the contribution of methyl syringate to the 106/7.3 ppm signal, which was estimated from the integral of its characteristic signal at 52/3.8 ppm.

Abbreviations

CEL, cellulolytic enzyme lignin; DMSO-d_6, deuterated dimethylsufoxide; FPU, filter-paper units; G, guaiacyl; HBT, 1-hydroxybenzotriazole; HSQC, heteronuclear single-quantum correlation; NMR, nuclear magnetic resonance; Py-GC/MS, Pyrolysis gas chromatography/mass spectrometry; S, syringyl.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

AR carried out all of the experimental work presented here, including the enzymatic pretreatments, saccharification assays and GC analyses and lignin isolation. JR conducted the 2D-NMR and Py-GC/MS analyses including data interpretation. JCR contributed in pyrolysis data interpretation. ATM substantially contributed to the 2D-NMR data interpretation and quantification and was involved in critically reviewing the manuscript with substantial contribution to its intellectual content. AG conceived of the study, supervised the work, substantially contributed to analysis and interpretation of data and wrote the manuscript. All authors read and approved the final manuscript.

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PUBLICACIÓN 4:

In-depth 2D NMR study of lignin modification during pretreatment of *Eucalyptus* wood with laccase and mediators.

Alejandro Rico, Jorge Rencoret, José C. del Río, Angel T. Martínez and Ana Gutiérrez.

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In-depth 2D NMR study of lignin modification during pretreatment of *Eucalyptus* wood with laccase and mediators

Alejandro Rico[a], Jorge Rencoret[a], José C. del Río[a], Angel T. Martínez[b], Ana Gutiérrez[a]

[a]Instituto de Recursos Naturales y Agrobiología de Sevilla, CSIC, Reina Mercedes 10, E-41012 Seville, Spain

[b]Centro de Investigaciones Biológicas, CSIC, Ramiro de Maeztu 9, E-28040 Madrid, Spain

Abstract

Eucalypt feedstock was pretreated with *Myceliophthora thermophila* laccase, and methyl syringate as mediator, in a multistage sequence consisting of successive enzymatic and alkaline peroxide stages, directly on the ground wood. Lignin modification was studied by 2D-NMR of wood (at the gel stage) after each stage of the sequence. Decrease of guaiacyl and syringyl lignin units (with preferential removal of the former) and aliphatic (mainly β-*O*-4'-linked) side-chains, without a substantial change in polysaccharide cross-signals along the sequence, was observed. However, the most noticeable modification was the formation of C_α-oxidized syringyl units in the enzymatic stages, which were partially removed in the alkaline extraction. Further insight was attained by 2D-NMR of the cellulolytic-enzyme lignins isolated from the pretreated samples, and the filtrates from the different stages. Additionally, a comparison of eucalypt delignification with the high redox-potential laccase from *Pycnoporus cinnabarinus* in the presence of the synthetic mediator 1-hydroxybenzotriazole was carried out. Wood delignification already at the first cycle of the pretreatment with *P. cinnabarinus* laccase and 1-hydroxybenzotriazole, which directly correlated with increases in glucose (~10%) yields after enzymatic hydrolysis, was observed. In contrast, the first cycle pretreatment with *M. thermophila* laccase and methyl syringate did not produce any delignification or saccharification improvement. However, at the end of the sequence, similar delignification (~50%) of eucalypt feedstock and increases (~40%) in glucose yields were attained in both laccase-mediator pretreatments. Finally, the influence of lignin content and the ratio of its syringyl and guaiacyl units

on recalcitrance of eucalypt feedstock to enzymatic saccharification is discussed.

Keywords: Enzymatic delignification, Laccase-mediator, Lignin, S/G ratio, 2D-NMR, Bioethanol

1. Introduction

Bioconversion of lignocellulosic biomass is an attractive route for the sustainable production of liquid transportation fuels. However, the conversion of lignocellulosic biomass, constituted mainly by cellulose, hemicelluloses and lignin linked into a complex matrix, is challenged by its recalcitrant structure. Biomass recalcitrance towards saccharification correlates with the content and composition of lignin [1-3]. Lignin is a three-dimensional polymer constituted by phenylpropanoid subunits linked together by a variety of ether and carbon-carbon bonds. Lignin is intimately interlaced with hemicelluloses in the plant cell wall forming a matrix to cover the crystalline cellulose microfibrils. Its aromatic nature and complex structure makes lignin degradation very difficult. Lignin has been shown to have a detrimental effect on the hydrolysis of biomass because it physically hinder the accessibility of cellulases and also binds cellulases leading to their inactivation [4-7].

Biofuel production from lignocellulosic material requires deconstruction of the cell-wall into individual constituents, and hydrolysis of the carbohydrates into monomeric sugars followed by fermentation to ethanol. Biomass pretreatment is intended to increase enzyme accessible cellulosic surface area, that can be accomplished through the removal of lignin, and biotechnology can contribute to plant biomass deconstruction by providing biocatalysts being able to degrade or modify lignin [8]. Among these, laccases are promising enzymes with great biotechnological potential. Laccases (phenoloxidases, EC 1.10.3.2) are multicopper oxidases that oxidize substituted phenols using molecular oxygen as the final electron acceptor. The direct action of laccases on lignin is, in principle, restricted to phenolic units that only represent a small percentage of the total polymer, a fact that limits their biotechnological application. However, the discovery that some synthetic compounds can act as electron carriers between the enzyme and the final substrate [9], 1-hydroxybenzotriazole (HBT) being among the most efficient ones [10], has expanded the utility of laccases. A number of studies have confirmed the potential of laccase-mediator systems for paper pulp delignification [11,12], pitch control [13], polymer modification [14], and other applications in the forest industry [15]. The ability of high

redox-potential laccases (e.g. from *Trametes* species) to remove lignin (when applied in combination with synthetic mediators, based on nitrogen heterocycles) from whole [16] or pretreated [17,18] lignocellulosic biomass, making cellulose accessible to hydrolysis, was reported.

Recently, a low redox-potential laccase from the ascomycete *Myceliophthora thermophila* in combination with the natural mediator methyl syringate (MeS) was tested for the removal of lignin from *Eucalyptus globulus* wood feedstock [19]. After a multistage sequence, up to 50% lignin removal was attained that was directly correlated with increases (approximately 40%) in glucose and xylose yields after enzymatic hydrolysis. In the present work, a thorough two-dimensional nuclear magnetic resonance (2D-NMR) study of lignin modification after each step of the *Eucalyptus* wood pretreatment multistage sequence, consisting of successive *M. thermophila* laccase and MeS and alkaline peroxide stages, has been performed. Both the pretreated eucalypt wood and the corresponding filtrates obtained after each stage of pretreatment have been analyzed by 2D-NMR, together with the cellulolytic enzyme lignin (CEL) isolated from the pretreated wood. Additionally, a comparison of eucalypt wood delignification with the high redox potential laccase from *Pycnoporus cinnabarinus* in presence of the synthetic mediator HBT and its effect on saccharification yield was also carried out along the several stages of the sequence. The aim of the present study is not to develop a cost effective pretreatment but to provide detailed chemical information about the eucalypt lignin modification by a commercial laccase and a phenolic mediator (as well as by the subsequent alkaline peroxide extraction) in the course of a model multistage sequence, and the effect of lignin removal on enzymatic hydrolysis, that will help to develop economically viable pretreatments.

2. Material and methods

2.1. Wood, enzyme and mediator

Eucalypt (*E. globulus*) wood chips from ENCE (Pontevedra, Spain), were air-dried and grounded in an IKA MF10 cutting mill to pass through a 100-mesh screen and then finely milled using a Retsch PM100 planetary mill at 400 rev·min^{-1} (with 5 min breaks after every 5 min of milling) using a 500 mL agate jar and agate ball bearings (20×20 mm). The total ball-milling time for the samples was 5 h.

A commercial (recombinant) fungal laccase from the ascomycete *M. thermophila*, provided by Novozymes (Bagsvaerd, Denmark), was used in this study. Its activity was measured as initial velocity during oxidation of

5 mM ABTS from Roche to its cation radical (ε_{436} 29300 $M^{-1}\cdot cm^{-1}$) in 0.1 M sodium acetate (pH 5) at 24ºC. The laccase activity of the enzyme preparation was 945 U/ml. One activity unit (U) was defined as the amount of enzyme transforming 1 µmol of ABTS per min. A fungal laccase preparation (45 U/ml) obtained from a laccase-hyperproducing strain of the basidiomycete *Pycnoporus cinnabarinus* that was provided by Beldem (Andenne, Belgium) was also used for comparative studies.

MeS (methyl 4-hydroxy-3,5-dimethoxybenzoate) from Alfa Aesar (Karlsruhe, Germany) and 1-hydroxybenzotriazol (HBT) from Sigma-Aldrich (Steinheim, Germany) were used as mediators.

2.2. Laccase-mediator treatments

The eucalypt samples were treated with the *M. thermophila* laccase in the presence (and absence) of MeS, as mediator. Laccase doses of 50 $U\cdot g^{-1}$ were assayed, together with 3% MeS, both referred to wood dry weight. The treatments were carried out in 200 mL pressurized bioreactors (Labomat, Mathis) placed in a thermostatic shaker at 170 $rev\cdot min^{-1}$ and 50ºC, using 10 g (dry weight) samples at 6% consistency (w:w) in 50 mM sodium dihydrogen phosphate (pH 6.5) under O_2 atmosphere (2 bars) for 24 h. After the treatment, the samples were filtered through a Büchner funnel and washed with 1 L of water. In a subsequent stage, samples at 6% consistency (w:w) were submitted to a peroxide-reinforced alkaline extraction using 1% (w:w) NaOH and 3% (w:w) H_2O_2 (also with respect to sample dry weight) at 80ºC for 90 min, followed by water washing [20]. Cycles of four successive enzyme-extraction treatments were applied. Treatments with laccase (50 $U\cdot g^{-1}$) alone (without mediator) and controls without laccase and mediator, were also performed (followed in both cases by the corresponding alkaline extractions). Each filtrate from the pretreatment stages (including enzymatic treatments and alkaline peroxide extractions) were recovered and freeze-dried. Eucalypt treatments with *P. cinnabarinus* laccase were performed as described above for *M. thermophila* ones, but using 50 mM sodium tartrate (pH 4) as a buffer and HBT as mediator. Klason lignin content was estimated according to T222 om-88 [21].

2.3. Saccharification of treated wood

The laccase-pretreated samples were hydrolyzed with a cocktail containing commercial enzymes (from Novozymes, Bagsvaerd) with cellulase (Celluclast 1.5L; 2 $FPU\cdot g^{-1}$) and β-glucosidase (Novozym 188; 100 $nkat\cdot g^{-1}$) activities, at 1% consistency in 3 mL of 100 mM sodium citrate (pH 5) for 72 h at 45ºC, in a thermostatic shaker at 170 $rev\cdot min^{-1}$ (in triplicate experiments).

The different monosaccharides released were determined as alditol acetates [22] by GC. An Agilent 6890N Network GC system equipped with a split−splitless injector and a flame ionization detector was used. The injector and detector temperatures were set at 225 and 250°C, respectively. Samples were injected in the split mode (split ratio 10:1). Helium was used as the carrier gas. The capillary column used was a DB-225 (30 m × 0.25 mm i.d., 0.15 μm film thickness; Agilent J&W). The oven was temperature-programmed from 220°C (held for 5 min) to 230°C (held for 5 min) at 2°C·min^{-1}. Peaks were quantified by area and glucose, xylose and arabinose were used as standards to elaborate calibration curves. The data from the three replicates were averaged.

2.4. Isolation of cellulolytic enzyme lignin (CEL)

The air-dried eucalypt samples were extracted three times with water and subsequently three times with 80% ethanol by sonicating in an ultrasonic bath for 30 min each time. CEL preparations were isolated by enzymatically saccharifying polysaccharides as described by Chang et al. (1975). Cellulysin cellulase (Calbiochem), a crude cellulase preparation from *Trichoderma viride* also containing hemicellulase activities, was used. Its activity was ≥10 000 FPU·g^{-1} of dry weight. The extractives free ball-milled material (200 mg) was suspended in 30 ml of 20 mM sodium acetate (pH 5.0) in a 50 ml centrifuge tube, 7.5 mg of Cellulysin was added, and the reaction slurry was incubated at 30ºC for 48 h. The solids were pelleted by centrifugation (8000 rpm, 4ºC, 20 min), and the process was repeated with fresh buffer and enzyme, three times. Finally, the residue (CEL) was washed with distillated water, recovered by centrifugation and freeze dried.

2.5. 2D-NMR spectroscopy

For gel-state NMR experiments, ~100 mg of finely divided (ball-milled) extractive-free wood samples (and filtrate samples) after the several steps of the whole multistage sequence were directly transferred into 5-mm NMR tubes, and swelled in 1 mL of DMSO-d_6, forming a gel inside the NMR tube [23,24]. For a more in depth structural characterization of the lignins, around 30 mg of CEL preparations were dissolved in 0.75 mL of DMSO-d_6.

HSQC 2D-NMR spectra were acquired at 25ºC on a Bruker AVANCE III 500 MHz spectrometer fitted with a cryogenically cooled 5 mm TCI gradient probe with inverse geometry (proton coils closest to the sample). The 2D ^{13}C-^{1}H correlation spectra were carried out using an adiabatic HSQC pulse program (Bruker standard pulse sequence 'hsqcetgpsisp2.2') and the following parameters: spectra were acquired from 10 to 0 ppm (5000 Hz) in F2 (^{1}H) using 1000 data points for an

acquisition time (AQ) of 100 ms, an interscan delay (D1) of 1 s, and from 200 to 0 ppm (25,168) in F1 (^{13}C) using 256 increments of 32 scan, for a total acquisition time of 2 h 34 min. The $^{1}J_{CH}$ used was 145 Hz. Processing used typical matched Gaussian apodization in ^{1}H and a squared cosine bell in ^{13}C. The central solvent peak was used as an internal reference (δ_C/δ_H 39.5/2.49). The ^{13}C-^{1}H correlation signals from the aromatic region of the spectrum were used to estimate the lignin composition in terms of G, S and oxidized S (S') units, and those of the aliphatic-oxygenated region were used to estimate the inter-unit linkage and end-unit abundances. The S lignin content in the laccase-mediator treated sample was corrected for the contribution of MeS to the 106/7.3 ppm signal, which was estimated from the integral of its characteristic signal at 52/3.8 ppm.

3. Results

The purpose of the present work is to analyze in detail the chemical modifications produced in eucalypt feedstock and its recalcitrant lignin moiety after each step of the multistage sequence consisting of four successive enzymatic treatments with the commercial *M. thermophila* laccase and mediator, followed by alkaline peroxide extraction stages. Additionally, the efficiency of this low redox potential laccase and the MeS mediator in lignin removal and improvement in saccharification yield after enzymatic hydrolysis was compared with that of a high redox potential laccase and a synthetic mediator along the multistage sequence.

Samples of the whole lignocellulosic material (and also their isolated CEL lignins) including controls, samples pretreated with laccases alone (from *M. thermophila* and *P. cinnabarinus*) and samples pretreated with the two laccases and mediators (MeS and HBT, respectively) and filtrates recovered after each stage of the sequence were thoroughly studied by 2D-NMR. Semi-quantitative data on lignin modification during the multi-stage sequence were obtained by volume integrals of aromatic and aliphatic lignin signals (providing information on the amount of lignin S and G units, and side-chain interunit linkages, respectively) in the above 2D-NMR spectra, which were obtained using an adiabatic pulse program that largely corrects previous intensity problems due to differences in $^{1}J_{CH}$ values and magnetization losses (offset effects) in different regions of the HSQC spectrum.

3.1. Lignin modification along the *M. thermophila* laccase-MeS pretreatment

3.1.1. 2D-NMR analyses of the whole pretreated samples

The modification of lignin structure during the laccase-mediator treatment of eucalypt wood was studied after each of the 8 stages, including 4 enzymatic treatments under oxygen (stages 1,3,5 and 7) followed each of them by an alkaline peroxide extraction (stages 2, 4, 6 and 8). With this purpose, all the pretreated eucalypt samples (and the corresponding controls) were analyzed by 2D-NMR at the gel state (**Figs. 1-3, Table 1**). The initial wood sample (without any treatment) was also analyzed. The main lignin structures identified are shown in **Fig. 4**, and the different lignin signals assigned on the spectra are listed in **Table 2.** The aliphatic-oxygenated region of the HSQC spectrum of the initial eucalypt wood (**Fig. 1a**, top-right) shows signals of lignin and carbohydrates, the latter mainly corresponding to xylan (X) and acetylated xylan (X') units, since crystalline cellulose is nearly "silent" in lignocellulose gel spectra under solution NMR conditions. In this region, signals of side-chains in β-*O*-4' alkyl-aryl ether lignin substructures (A), including C_γ-H_γ, C_β-H_β and C_α-H_α correlations (A_γ, A_β and A_α, respectively) were observed. The A_γ signal overlaps with related signals in lignin and other lignocellulose constituents. The C_β-H_β correlations gave two different signals corresponding to β-*O*-4' substructures where the second unit is an S unit or a G unit ($A_{\beta(S)}$ and $A_{\beta(G)}$), the latter with lower intensity, in agreement with lignin composition described below. Other less prominent signals for resinol (β–β') substructures (C) were also observed in the spectrum, with their C_α–H_α, C_β–H_β and the double C_γ–H_γ correlations (C_α, C_β and C_γ). The main signals in the aromatic region of the HSQC spectrum (**Fig. 1a**, bottom-left) correspond to the benzenic rings of the S and G lignin units. The S-lignin units showed a prominent signal for the $C_{2,6}$-$H_{2,6}$ correlation ($S_{2,6}$), while the G-lignin units showed different correlations for C_2-H_2 (G_2), C_5-H_5 (G_5) and C_6-H_6 (G_6). Signals corresponding to $C_{2,6}$-$H_{2,6}$ correlations in C_α-oxidized S-lignin units ($S'_{2,6}$) were also observed although in low amount. From the integrals of the above signals an S/G ratio around 3.5, and a large predominance of β-*O*-4' ether linkages, together with some resinols, were estimated for lignin in *E. globulus* wood (**Table 1**).

Table 1. Lignin units (S, G and S' molar percentages) and interunit linkages (side-chains per 100 units) from the HSQC spectra of eucalypt samples from a multistage enzymatic sequence, including 4 treatments with *M. thermophila* laccase-MeS and 4 alkaline peroxide extractions, compared with a control without enzyme and a treatment with laccase alone.

	Initial	Whole treated wood (different stages)							
	wood	1	2	3	4	5	6	7	8
1) Control									
- Lignin units:									
Syringyl (S) (% total)	78	74	75	76	75	76	76	79	79
Guaiacyl (G) (% total)	22	26	25	24	25	24	24	21	21
C-oxidized S units (S') (% S)	12	10	8	9	9	7	12	10	10
S/G ratio	3.5	2.8	2.9	3.1	2.9	3.2	3.3	3.8	3.7
- Lignin interunit linkages (% S+G):									
β-*O*-4' Alkyl-aryl ethers (**A**)	64	58	58	56	51	54	53	45	50
Phenylcoumarans (**B**)	0	1	0	0	0	0	0	0	0
Resinols (**C**)	10	10	8	9	5	6	7	5	5
Spirodienones (**D**)	0	1	1	0	0	1	0	0	0
Total	74	71	67	65	57	60	60	50	54
2) *M. thermophila* laccase									
- Lignin units:									
Syringyl (S) (% total)		78	78	80	78	83	84	84	86
Guaiacyl (G) (% total)		22	22	20	22	17	16	16	14
C-oxidized S units (S') (% S)		13	11	14	12	19	13	18	16
S/G ratio		3.5	3.5	4	3.5	4.7	5.3	5.3	6.2
- Lignin interunit linkages (% S+G):									
β-*O*-4' Alkyl-aryl ethers (**A**)		58	54	46	48	43	45	46	43
Phenylcoumarans (**B**)		0	0	0	0	0	0	0	0
Resinols (**C**)		9	5	2	5	3	2	0	0
Spirodienones (**D**)		0	0	0	0	0	0	0	0
Total		68	59	48	53	46	47	46	43
3) *M. thermophila* laccase-MeS									
- Lignin units:									
Syringyl (S) (% total)		86	84	96	94	100	100	100	100
Guaiacyl (G) (% total)		14	16	4	6	0	0	0	0
C-oxidized S units (S') (% S)		44	26	64	37	65	46	67	47
S/G ratio		6.2	5.4	25.2	15.2	----	----	----	----
- Lignin interunit linkages (% S+G):									
β-*O*-4' Alkyl-aryl ethers (**A**)		40	46	24	33	19	31	32	24
Phenylcoumarans (**B**)		0	0	0	0	0	0	0	0
Resinols (**C**)		6	8	1	1	0	0	0	0
Spirodienones (**D**)		0	0	0	0	0	0	0	0
Total		46	54	25	34	19	31	32	24

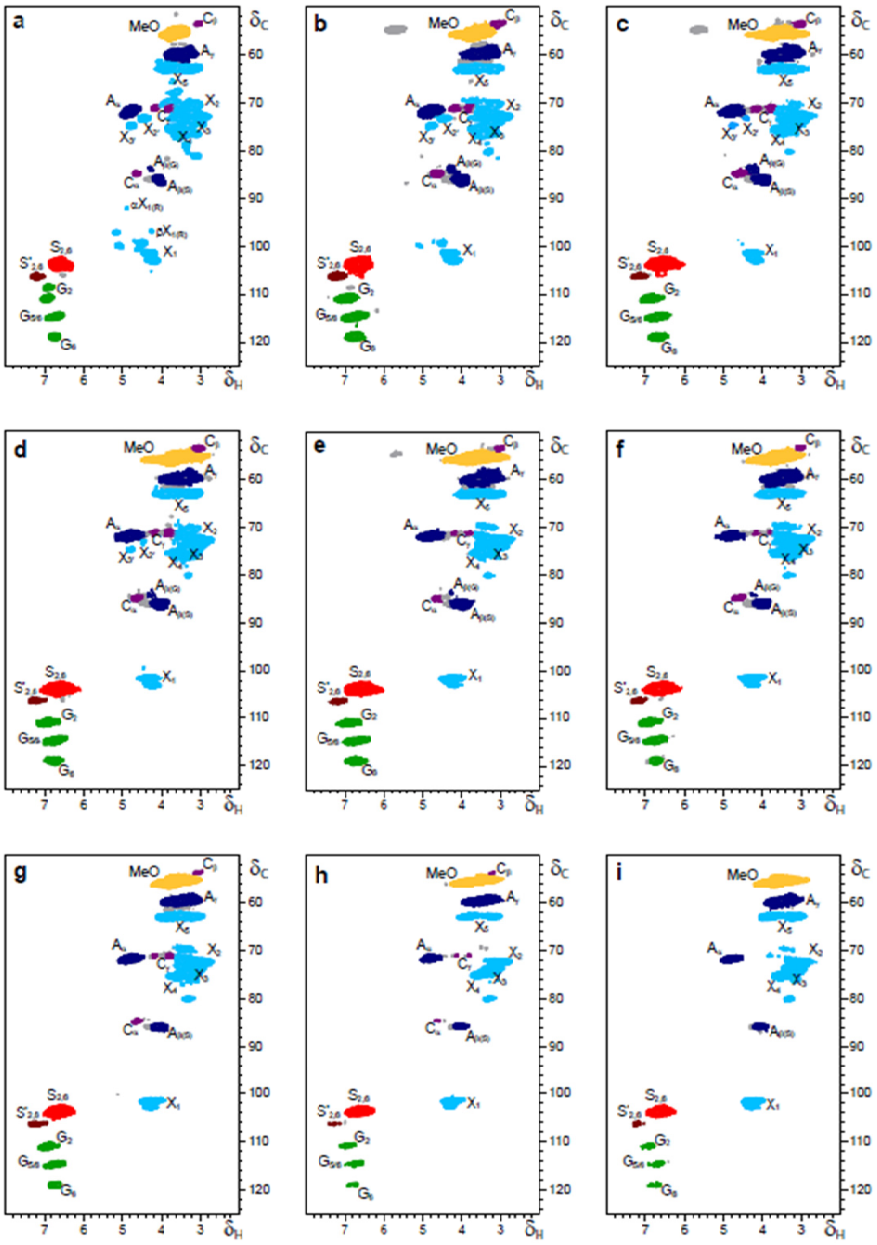

Fig. 1. HSQC NMR spectra of whole eucalypt wood (control samples without enzyme) swollen in dimethylsulfoxide-d_6, from a multistage enzymatic sequence, including 4 treatments with *M. thermophila* laccase-MeS and 4 alkaline peroxide extractions: **a**) Initial eucalypt sample; **b**), **d**), **f**) and **h**) Controls of first, second, third and fourth enzymatic pretreatment (stages 1, 3, 5 and 7, respectively); **c**), **e**), **g**) and **i**) Controls of first, second, third and fourth alkaline peroxide extractions (stages 2, 4, 6 and 8, respectively). See **Table 1** for quantification of the lignin structures identified, **Table 2** for signal assignment, and **Fig. 4** for the main lignin structures identified.

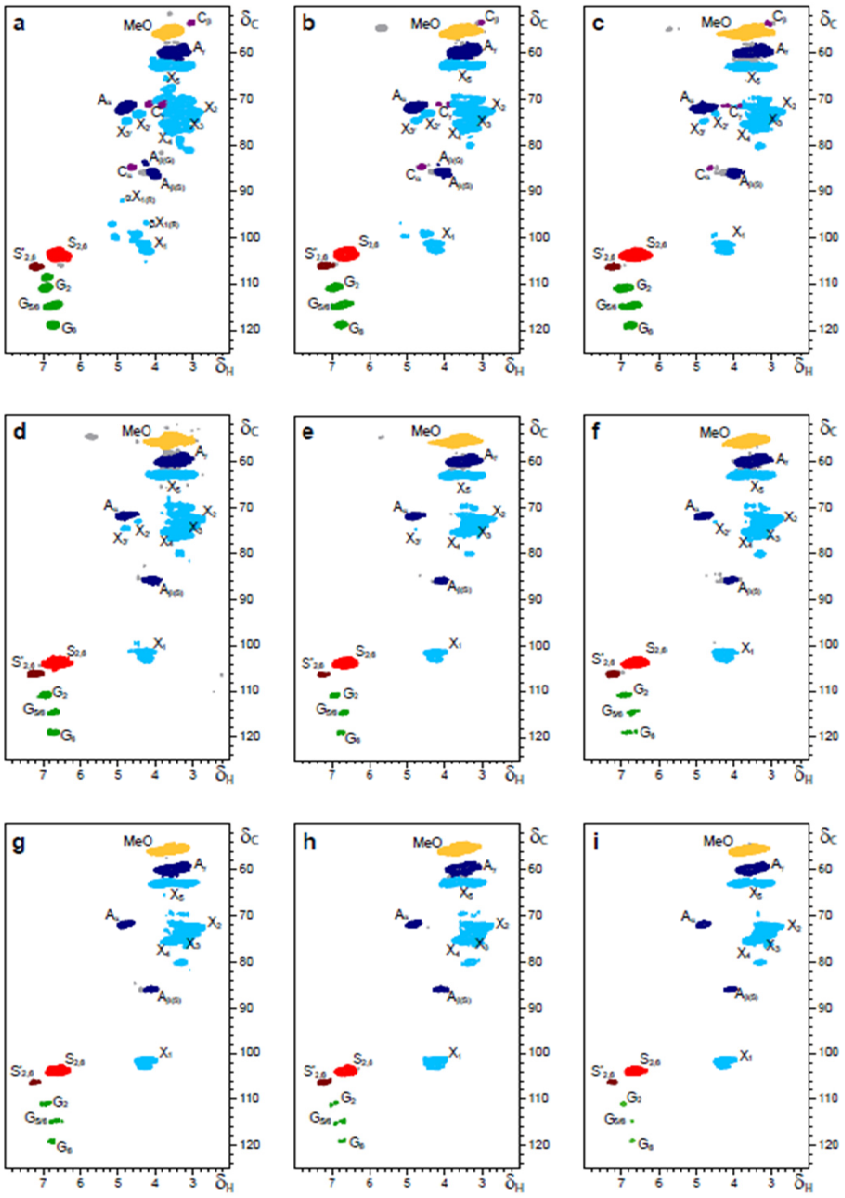

Fig. 2. HSQC NMR spectra of whole eucalypt wood (samples treated with *M. thermophila* laccase alone) swollen in dimethylsulfoxide-d_6, from a multistage enzymatic sequence, including 4 treatments with *M. thermophila* laccase-MeS and 4 alkaline peroxide extractions: **a**) Initial eucalypt sample; **b**), **d**), **f**) and **h**) Samples from first, second, third and fourth enzymatic pretreatment with laccase alone (stages 1, 3, 5 and 7, respectively); **c**), **e**), **g**) and **i**) Samples from first, second, third and fourth alkaline peroxide extractions (stages 2, 4, 6 and 8, respectively). See **Table 1** for quantification of the lignin structures identified, **Table 2** for signal assignment, and **Fig. 4** for the main lignin structures identified.

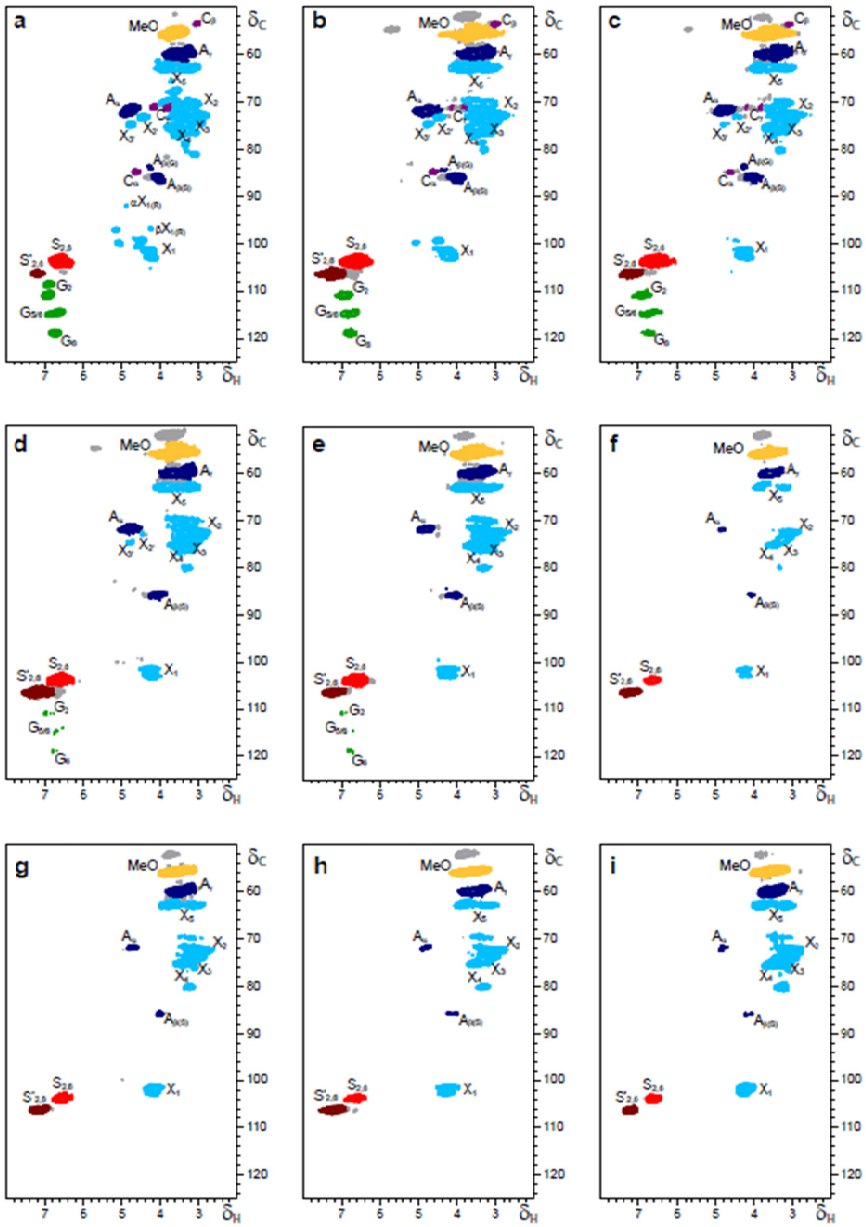

Fig. 3. HSQC NMR spectra of whole eucalypt (samples treated with *M. thermophila* laccase and MeS) swollen in dimethylsulfoxide-d_6, from a multistage enzymatic sequence, including 4 treatments with *M. thermophila* laccase-MeS and 4 alkaline peroxide extractions: **a**) Initial eucalypt sample; **b**), **d**), **f**) and **h**) Samples from first, second, third and fourth enzymatic pretreatment with laccase and mediator (stages 1, 3, 5 and 7, respectively); **c**), **e**), **g**) and **i**) Samples from first, second, third and fourth alkaline peroxide extractions (stages 2, 4, 6 and 8, respectively). See **Table 1** for quantification of the lignin structures identified, **Table 2** for signal assignment, and **Fig. 4** for the main lignin structures identified.

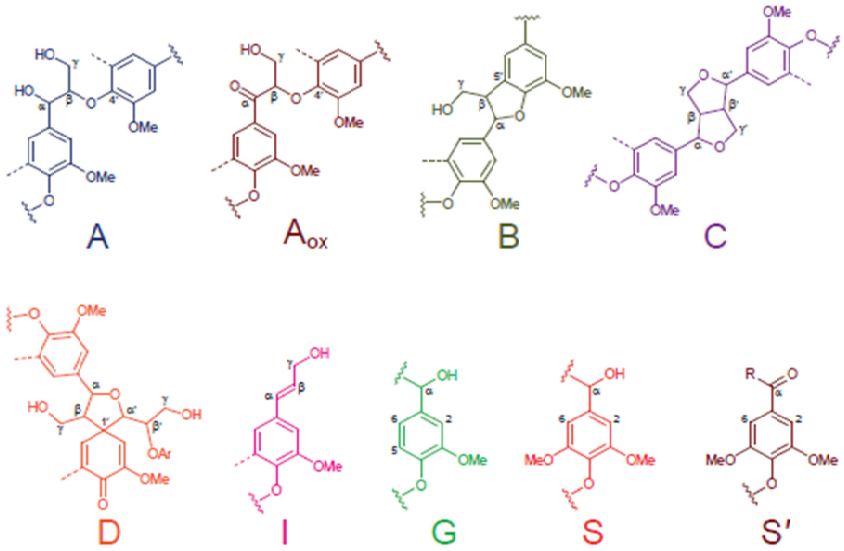

Fig. 4. Main lignin structures identified in the eucalypt samples analyzed by HSQC NMR (**Figs. 1-3** and **5-7**): (**A**) β-*O*-4' lignin substructures (including a second S or G unit); (**Aox**) C_α-oxidized β-*O*-4' lignin substructures (including a second S or G unit); (**B**) phenylcoumarans; (**C**) resinols; (**D**) spirodienones; (**I**) cinnamyl alcohol end-groups; (**G**) guaiacyl units; (**S**) syringyl units; and (**S'**) C_α-oxidized S units (R can be a hydroxyl in carboxylic acids or a lignin side-chain in ketones).

Figure 1(b-i) shows the HSQC spectra of the eucalypt controls (treated under the same conditions of the laccase-mediator treatments but without laccase and mediator). In the aliphatic oxygenated region of the spectra it can be observed that during the pretreatment acetylated xylan units (X´) decrease considerably after the first alkaline extraction (stage 2, **Fig. 1c**) and completely disappeared with the second alkaline extraction (stage 4) while the non-acetylated ones (X) remain. The β-*O*-4' alkyl-aryl ethers and resinol substructures decreased slightly along the sequence and after the last stage the lignin in control were mainly constituted by the former substructures although the latter were still present (**Table 1**). The most significant effect of the control pretreatment conditions was the increase in the S/G ratio along the sequence that particularly took place in the oxygen stages and was especially evident in the stage 7.

The 2D-NMR analyses of gels of the eucalypt samples treated with *M. thermophila* laccase alone (followed by alkaline treatments) (**Fig. 2**, **Table 1**) revealed a decrease in the β-*O*-4' alkyl-aryl ethers and resinol substructures along the sequence showing the sharpest decrease in the second enzymatic treatment (stage 3). Concerning lignin units an

increase in the S/G ratio was observed along the sequence, that was especially significant along the third and fourth cycles of treatment. One effect of the enzymatic treatment with *M. thermophila* laccase (not observed along the control sequence) was the increase in C_α-oxidized lignin units (S') produced in the enzymatic stages 3, 5 and 7.

However, the most noticeable changes were observed in the pretreatment of eucalypt wood with *M. thermophila* laccase in the presence of MeS (**Fig. 3, Table 1**). This included the complete absence of G units and resinol substructures, together with a high decrease in β-*O*-4' alkyl-aryl ethers, after 2 cycles of laccase-mediator treatment and alkaline extraction (stage 5), although the most remarkable decreases in these units and substructures were already observed after the first cycle of treatment (stage 3) at the same time that the signals corresponding to β-*O*-4' substructures where the second unit is a G unit ($A_{\beta(G)}$) dissapeared (**Fig. 3d**). Likewise, the most remarkable increase in C_α-oxidized lignin units (S') was also observed in stage 3. Interestingly, the amount of these oxidized lignin units decreased in all cases with the alkaline extraction (stages 2, 4, 6 and 8).

Table 2. Assignments of lignin main ^{13}C-^{1}H correlation signals in the HSQC spectra of the whole wood, CEL and filtrate samples (in dimethylsulfoxide-d_6). See **Fig. 4** for chemical structures.

Label	δ_C/δ_H (ppm)	Assignment
B_β	53.1/3.45	C_β−H_β in phenylcoumaran substructures (**B**)
C_β	53.3/3.05	C_β−H_β in β−β′ resinol substructures (**C**)
MeO	55.6/3.72	C−H in methoxyls
A_γ	59.4 /3.38 and 3.70	C_γ−H_γ in β-*O*-4' structures (**A**)
D_β	59.7/2.73	C_β−H_β in spirodienone substructures (**D**)
I_γ	61.3/4.08	C_γ−H_γ in cinnamyl alcohol end-groups (**I**)
B_γ	62.6/3.67	C_γ−H_γ in phenylcoumaran substructures (**B**)
A_α	71.8/4.85	C_α−H_α in β-*O*-4' structures (**A**)
C_γ	71.0/ 3.81 and 4.18	C_γ−H_γ in β−β′ resinol substructures (**C**)
$D_{\beta'}$	79.2/4.10	$C_{\beta'}$−$H_{\beta'}$ in spirodienone substructures (**D**)
D_α	81.0/5.08	C_α−H_α in spirodienone substructures (**D**)
$A_{ox\beta}$	83.0/5.20	C_β−H_β in α-oxidized β-O-4′ substructures (**A_{ox}**)
$A_{\beta(G)}$	83.6/4.28	C_β−H_β in β-*O*-4' structures (**A**) linked to a G-unit
$D_{\alpha'}$	83.7/4.68	$C_{\alpha'}$−$H_{\alpha'}$ in spirodienone substructures (**D**)
C_α	84.7/4.64	C_α−H_α in β−β′ resinol substructures (**C**)
$A_{\beta(S)}$	85.7/4.10	C_β−H_β in β-*O*-4' structures (A) linked to a S-unit
B_α	86.4/5.43	C_α−H_α in phenylcoumaran substructures (**B**)
$S_{2,6}$	103.9/6.69	C_2−H_2 and C_6−H_6 in syringyl units (**S**)
$S'_{2,6}$	106.1/7.31	C_2−H_2 and C_6−H_6 in α-oxidized syringyl units (**S'**)
G_2	110.8/6.96	C_2−H_2 in guaiacyl units (**G**)
D_2	113.3/6.25	C_2−H_2 in spirodienone substructures (**D**)
G_5/G_6	114.3/6.69 and 114.9/6.94	C_5−H_5 and C_6−H_6 in guaiacyl units (**G**)
G_6	118.8/6.78	C_6−H_6 in guaiacyl units (**G**)
D_6	118.7/6.06	C_6−H_6 in spirodienone substructures (**D**)

3.1.2. 2D-NMR analyses of the lignins isolated from the pretreated samples

To gain more insight into the modification of lignin structure along the multistage laccase-mediator treatment sequence, CEL was isolated from the pretreated eucalypt samples, and analyzed by 2D-NMR (**Fig. 5**). The lignin structures identified, some of them not detected in the wood spectra, are shown in **Fig. 4**, and the corresponding signals are listed in **Table 2**, together with those found in the whole wood spectra. **Table 3** shows the lignin composition, and percentages of inter-unit linkages in the CEL samples at the beginning (cycle 1) and at the end (cycle 4) of pretreatment (stages 2 and 8, respectively) estimated from the signal volume integrals of 2D-NMR spectra as described for the wood spectra.

The HSQC spectrum of CEL preparations from control samples after 1 and 4 cycles of treatment (**Fig. 5a-b**) showed the same side-chain signals observed in the spectrum of the corresponding whole eucalypt control samples (stages 2 and 8) (**Fig. 1c** and **i**), corresponding to β-*O*-4' alkyl-aryl ether (A) and resinol (C) correlations, although better resolved and with higher intensity, and several new signals that could not be observed in the wood spectra. The latter included: **i)** spirodienone (β-1'/α-O-α') substructures (D) with their C_{α}-H_{α}, $C_{\alpha'}$-$H_{\alpha'}$, C_{β}-H_{β}, and $C_{\beta'}$-$H_{\beta'}$ correlations (D_{α}, $D_{\alpha'}$, D_{β}, $D_{\beta'}$); **ii)** small signals corresponding to phenylcoumaran (β-5') substructures (B) with their C_{α}-H_{α}, C_{β}-H_{β}, and C_{γ}-H_{γ} correlations (B_{α}, B_{β} and B_{γ}; the two latter correlations overlapping with other signals); **iii)** a signal of cinnamyl alcohol end-groups (I) with its C_{γ}-H_{γ} correlation; and **iv)** signals of C_{β}-H_{β} correlations in C_{α}-oxidized β-*O*-4' alkyl-aryl ether substructures ($A_{ox\beta}$) (**Fig. 4**). The main signals in the aromatic region of the spectrum of the initial eucalypt CEL sample corresponded to the benzenic rings of the S and G lignin units as shown for the whole wood spectrum. Signals from C_{α}-oxidized S-lignin units ($S'_{2,6}$) were also observed. Some new signals not observed in the whole wood spectra appeared in this region corresponding to the above-mentioned spirodienone substructure (D) with $C_{2'}$-$H_{2'}$ and $C_{6'}$-$H_{6'}$ correlations ($D_{2'}$ and $D_{6'}$). Most of the lignin side chains in control samples were barely modified along the sequence as shown in **Table 3** for resinol, spirodienones and β-*O*-4' alkyl-aryl ether substructures. Only the phenylcoumarans decreased from cycle 1 to 4. The most remarkable effect of the control pretreatment conditions was the increase in the S/G ratio from 3.6 (stage 2) to 4.3 (stage 8) in agreement with that observed with the whole control samples.

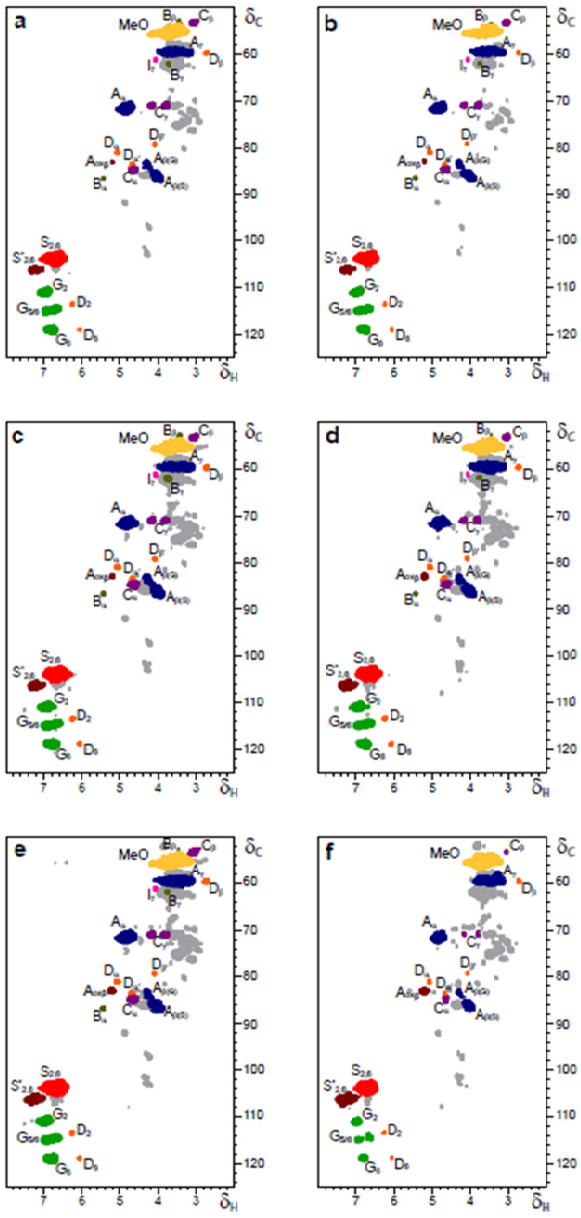

Fig. 5. HSQC NMR spectra of CEL lignins isolated from eucalypt wood samples from a multistage enzymatic sequence, including 4 treatments with *M. thermophila* laccase-MeS and 4 alkaline peroxide extractions: **a**) and **b**) Controls of first and fourth cycle of enzymatic pretreatment (stages 2 and 8, respectively); **c**) and **d**) Samples from first and fourth cycle of enzymatic pretreatment with laccase alone (stages 2 and 8, respectively); **e**) and **f**) Samples from first and fourth cycle of enzymatic pretreatment with laccase-MeS (stages 2 and 8, respectively). See **Table 2** for signal assignment, **Table 3** for quantification of the lignin structures identified, and **Fig. 4** for the main lignin structures identified.

Table 3. Lignin units (S, G and S' molar percentages) and interunit linkages (side-chains per 100 units) from the HSQC spectra of CEL samples from eucalypt wood at two stages (2 and 8) of a multistage sequence, including 4 treatments with *M. thermophila* laccase-MeS and 4 alkaline peroxide extractions, compared with a control without enzyme and a treatment with laccase alone.

	Initial wood	CEL	
		stage 2	stage 8
1) Control			
- *Lignin units:*			
Syringyl (S) (% total)	77	78	81
Guaiacyl (G) (% total)	23	22	19
C-oxidized S units (S') (% S)	6	8	10
S/G ratio	3.4	3.6	4.3
- *Interunit linkages and end-groups:*			
β-*O*-4' Alkyl-aryl ethers (**A**) (% S+G)	58	62	63
Phenylcoumarans (**B**) (% S+G)	2	2	1
Resinols (**C**) (% S+G)	9	8	8
Spirodienones (**D**) (% S+G)	2	2	2
Total (% S+G)	71	73	75
C_α-oxidized β-*O*-4' ethers ($\mathbf{A_{ox}}$) (% A)	2	2	2
2) *M. thermophila* laccase			
- *Lignin units:*			
Syringyl (S) (% total)		80	83
Guaiacyl (G) (% total)		20	17
C-oxidized S units (S') (% S)		10	17
S/G ratio		3.9	4.8
- *Interunit linkages and end-groups:*			
β-*O*-4' Alkyl-aryl ethers (**A**) (% S+G)		60	58
Phenylcoumarans (**B**) (% S+G)		2	1
Resinols (**C**) (% S+G)		7	6
Spirodienones (**D**) (% S+G)		2	2
Total (% S+G)		71	68
C_α-oxidized β-*O*-4' ethers ($\mathbf{A_{ox}}$) (% A)		2	5
3) *M. thermophila* laccase-MeS			
- *Lignin units:*			
Syringyl (S) (% total)		83	93
Guaiacyl (G) (% total)		17	7
C-oxidized S units (S') (% S)		15	34
S/G ratio		5.0	13.8
- *Interunit linkages and end-groups:*			
β-*O*-4' Alkyl-aryl ethers (**A**) (% S+G)		59	51
Phenylcoumarans (**B**) (% S+G)		2	0
Resinols (**C**) (% S+G)		7	2
Spirodienones (**D**) (% S+G)		2	1
Total (% S+G)		70	55
C_α-oxidized β-*O*-4' ethers ($\mathbf{A_{ox}}$) (% A)		5	13

The HSQC spectra of isolated lignins from the eucalypt samples after enzymatic pretreatment with *M. thermophila* laccase and MeS (stages 2 and 8) are shown in **Fig. 5 (e, f)** and the main differences in lignin units and inter-unit linkages, compared with the previous samples, are shown in **Table 3**. Concerning lignin composition, the most noticeable effect of the enzymatic treatments on the residual lignin remaining in wood was the significant reduction in G units produced by the laccase-mediator treatment resulting in an increase of the S/G ratio from 5.0 (stage 2) to 13.8 (stage 8). Additionally, a strong increase in C_α-oxidized S units was produced, as shown in **Table 3** where the contribution of MeS to the lignin $S'_{2,6}$ signal at 106/7.3 ppm was deduced. The increase of the $S'_{2,6}$ aromatic signal was accompanied by an increase in the C_β-H_β correlations signal from β-*O*-4' ether linked C_α-oxidized side chains ($A_{ox\beta}$). Moreover, a significant decrease in β-*O*-4' alkyl-aryl ether (A) and resinol substructures (C) per 100 phenylpropane units was the main effect observed in the side-chain region of the HSQC spectra of the lignin isolated from the wood treated with laccase and MeS, together with a decrease in the less intense signals of phenylcoumarans and spirodienones, which was especially evident in stage 8. This suggests a more degraded lignin than that present in control samples.

The 2D-NMR analysis of lignin isolated from eucalypt samples pretreated with laccase alone (**Fig. 5c,d**) also revealed some differences with respect to the control. Interestingly, the most remarkable effect is the increase of oxidized lignin structures evidenced by both aromatic ($S'_{2,6}$) and aliphatic side-chains ($A_{ox\beta}$) signals (**Table 3**) revealing that laccase alone attack lignin by a mechanism similar to that of the laccase-mediator system. On the other hand, in these samples a decrease in β-*O*-4' alkyl-aryl ether (A), spirodienones (D) and resinol substructures (C) was not observed in the side-chain region of the HSQC spectrum contrary to the laccase-mediator treated samples. This could be indicative of an oxidized lignin but less degraded than lignin in samples after laccase-mediator treatment.

3.1.3. 2D-NMR analyses of the filtrates from the pretreatment sequence

The filtrates obtained after each of the 8 steps of the multistage sequence were freeze-dried and analyzed by 2D-NMR to identify the degradation products of wood lignin after both enzymatic and alkaline peroxide extraction stages. **Table 4** shows the lignin composition of filtrates after the first (stage 2) and last cycle (stage 8) of pretreatment and **Fig. 6** shows the HSQC spectra of these filtrates. In addition to lignin, signals of carbohydrate (xylan) were also observed in the spectra. Comparing the characteristics of lignin in filtrates from stages 2 and 8, it can be observed that in control samples the S/G ratio increased and the percentage of interunit linkages identified in the HSQC spectra decreased from 73 to 69.

Table 4. Lignin units (S, G and S' molar percentages) and interunit linkages (side-chains per 100 units) from the HSQC spectra of the filtrates from eucalypt wood pretreatment at two stages (2 and 8) of a multistage sequence, including 4 treatments with *M. thermophila* laccase-MeS and 4 alkaline peroxide extractions, compared with a control without enzyme and a treatment with laccase alone.

	Filtrates	
	stage 2	stage 8
1) Control		
- Lignin units:		
Syringyl (S) (% total)	77	85
Guaiacyl (G) (% total)	23	15
C-oxidized S units (S') (% S)	12	10
S/G ratio	3.3	5.6
- Lignin interunit linkages (% S+G):		
β-*O*-4' Alkyl-aryl ethers (**A**)	59	62
Phenylcoumarans (**B**)	2	1
Resinols (**C**)	9	6
Spirodienones (**D**)	3	0
C_α-oxidized β-*O*-4' ethers ($\mathbf{A_{ox}}$) (% A)	0	0
Total	73	69
2) *M. thermophila* laccase		
- Lignin units:		
Syringyl (S) (% total)	83	92
Guaiacyl (G) (% total)	17	8
C-oxidized S units (S') (% S)	16	37
S/G ratio	4.8	11.5
- Lignin interunit linkages (% S+G):		
β-*O*-4' Alkyl-aryl ethers (**A**)	59	40
Phenylcoumarans (**B**)	1	0
Resinols (**C**)	8	0
Spirodienones (**D**)	2	0
C_α-oxidized β-*O*-4' ethers ($\mathbf{A_{ox}}$) (% A)	1	0
Total	70	40
3) *M. thermophila* laccase-MeS		
- Lignin units:		
Syringyl (S) (% total)	91	96
Guaiacyl (G) (% total)	9	4
C-oxidized S units (S') (% S)	47	41
S/G ratio	10.4	24.3
- Lignin interunit linkages (% S+G):		
β-*O*-4' Alkyl-aryl ethers (**A**)	39	32
Phenylcoumarans (**B**)	1	0
Resinols (**C**)	5	0
Spirodienones (**D**)	2	0
C_α-oxidized β-*O*-4' ethers ($\mathbf{A_{ox}}$) (% A)	2	0
Total	47	32

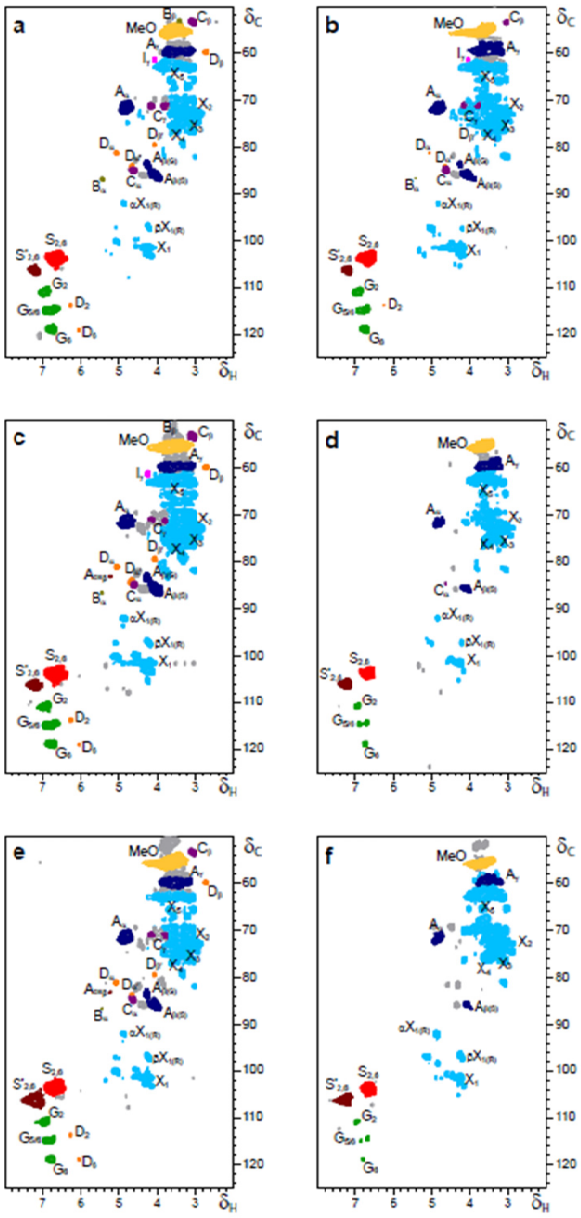

Fig. 6. HSQC NMR spectra of lignins from filtrates obtained from a multistage enzymatic sequence, including 4 treatments with *M. thermophila* laccase-MeS and 4 alkaline peroxide extractions: **a**) and **b**) Controls of first and fourth cycle of enzymatic pretreatment (stages 2 and 8, respectively); **c**) and **d)** Samples from first and fourth cycle of enzymatic pretreatment with laccase alone (stages 2 and 8, respectively); **e**) and **f**) Samples from first and fourth cycle of enzymatic pretreatment with laccase-MeS (stages 2 and 8, respectively). See **Table 2** for signal assignment, **Table 4** for quantification of the lignin structures identified, and **Fig. 4** for the main lignin structures identified.

This decrease was mainly due to the removal of phenylcoumarans, resinols and spirodienones substructures whereas the β-*O*-4' alkyl-aryl ether linkages remained. The increase in the S/G ratio and the loss of phenylcoumarans, resinols and spirodienones substructures from stages 2 to 8 was also observed in the lignin present in filtrates from laccase alone and laccase-mediator treatments. In contrast to control samples, a decrease in β-*O*-4' alkyl-aryl ether linkages was observed in the lignin present in filtrates from enzymatically treated samples. Also, a strong increase in C_α-oxidized S units was produced in the latter samples. It should be mentioned that in the spectra of filtrates from enzymatic stages (1, 3, 5 and 7) (data not shown) the presence of lignin signals were minor compared to alkaline peroxide stages (2, 4, 6 and 8) with syringyl units predominating.

3.2. Lignin modification along the *P. cinnabarinus* laccase-HBT pretreatment

Lignin modification during the pretreatment of eucalypt wood with a high redox potential laccase and mediator along a multistage sequence similar to that described above, was also studied by 2D-NMR with the objective of comparing the results with those obtained in the pretreatment of eucalypt wood with low redox potential laccase and mediator. With this purpose, 2D-NMR analyses of whole eucalypt samples (swollen in dimethylsulfoxide-d_6) after 1 and 4 cycles of pretreatment (stages 2 and 8, respectively) with *Pycnoporus cinnabarinus* laccase in the presence of the synthetic mediator HBT were carried out (**Fig. 7**) and the lignin composition, and percentages of inter-unit linkages in the whole samples are shown in **Table 5**. The qualitative composition in aromatic units and lignin interunit linkages of the residual lignins present in eucalypt wood after the whole sequence (stage 8) with the high and low redox potential laccases and their corresponding mediators was similar. Both HSQC spectra were characterized by the presence of syringyl units and the absence of guaiacyl ones. A considerable part of the syringyl units were oxidized in the C_α, reaching a 47% and 87% of total syringyl units in samples pretreated with *M. thermophila*-MeS and *P. cinnabarinus*-HBT, respectively. Likewise, the only interunit linkages present in these two lignins were the β-*O*-4' alkyl-aryl ether linkages although the proportions of these linkages were higher in the *M. thermophila*-MeS pretreated samples (24 per 100 phenylpropane units) than in the *P. cinnabarinus*-HBT ones (14 per 100 phenylpropane units). These results indicate that the latter lignin was more depolymerized and oxidized than the former. Similar tendencies were also obtained in the last stage of pretreatment of eucalypt wood with the two laccases alone (without mediator). In both cases, the β-*O*-4' linkages were the only interunit linkages observed in the HSQC spectra, representing a higher percentage in *M. thermophila* (43

per 100 phenylpropane units) than in *P. cinnabarinus* laccase (31 per 100 phenylpropane units). Likewise, lignins from both treatments were constituted mainly by syringyl units some of them being C_α-oxidized S units (S') with higher predominance in *P. cinnabarinus* laccase (28%) than in *M. thermophila* (16%). In addition, a relative low proportion of guaiacyl units were present in both lignins. The above results show that in spite of some quantitative differences both laccase mediator pretreatments modify lignin composition in a similar way after the whole multistage sequence.

Additionally, modification of eucalypt wood lignin with these two laccase-mediator systems was also studied at the beginning of the sequence (after one cycle of treatment). The 2D-NMR analyses of the whole samples revealed substantial differences between the two different laccase-mediator pretreatments at stage 2. The HSQC spectrum of the eucalypt wood pretreated with *P. cinnabarinus* laccase and HBT (**Fig. 7e**) showed a radically smaller amounts of lignin signals compared with that of *M. thermophila*-MeS (**Fig. 3c**) as revealed by the different amount of lignin interunit linkages per 100 phenylpropane units accounting for 19 and 54, respectively (**Table 5**) indicating that the former lignin was more degraded than the latter. Indeed, lignin from the *P. cinnabarinus*-HBT treatment is almost as degraded after the first cycle (19 per 100 phenylpropane units) as after the fourth one (14 per 100 phenylpropane units) unlike lignin from *M. thermophila*-MeS treatment. The main differences between lignins from both treatments at stage 2 relay on the composition in aromatic units that in the case of *P. cinnabarinus*-HBT treatment is constituted only by syringyl units while in the case of *M. thermophila*-MeS one is constituted by syringyl and guaiacyl ones. On the other hand, interunit linkages present in lignin from the latter treatment include both β-*O*-4' linkages and resinol substructures while lignin units from the former treatment are linked only by β-*O*-4' linkages.

The HSQC spectrum of the eucalypt wood pretreated with *P. cinnabarinus* laccase alone (**Fig. 7c**) (stage 2) also showed very smaller amounts of lignin signals compared with that of *M. thermophila* (**Fig. 2c**). Likewise, lignins from both treatments showed different amount of interunit linkages accounting for 30 and 59 per 100 phenylpropane units, respectively (**Table 5**) indicating that the former lignin was more degraded than the latter. Like in the case of laccase-mediator systems, lignin from the *P. cinnabarinus* treatment is equally degraded after the first and fourth cycle (about 30 per 100 phenylpropane units) unlike lignin from *M. thermophila* treatment (**Table 1**). With regard to lignin composition, samples from *P. cinnabarinus* and *M. thermophila* laccase treatment contained syringyl and guaiacyl units although in different proportions (S/G ratios of 6.2 and 3.5, respectively). Lignin interunit linkages included both β-*O*-4' linkages and resinol substructures in both treatments, resinol

amounts being similar in both lignins while β-*O*-4' linkages representing a higher amount in the *M. thermophila* laccase treatment.

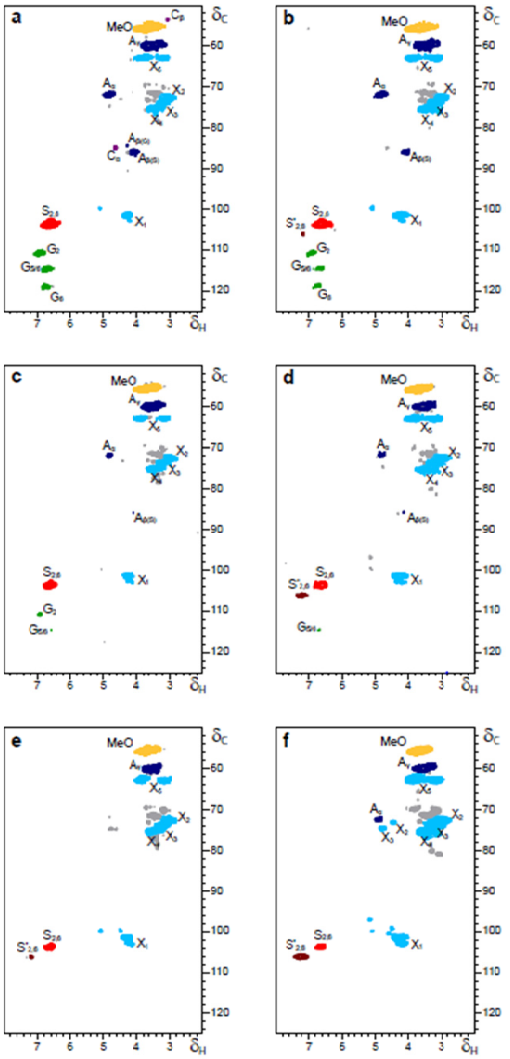

Fig. 7. HSQC NMR spectra of whole eucalypt wood (swollen in dimethylsulfoxide-d_6), from a multistage enzymatic sequence, including 4 treatments with *P. cinnabarinus* laccase-HBT and 4 alkaline peroxide extractions: **a**) and **b**) Controls of first and fourth cycle of enzymatic pretreatment (stages 2 and 8, respectively); **c**) and **d**) Samples from first and fourth cycle of enzymatic pretreatment with laccase alone (stages 2 and 8, respectively); **e**) and **f**) Samples from first and fourth cycle of enzymatic pretreatment with laccase and mediator (stages 2 and 8, respectively). See **Table 2** for signal assignment, **Table 5** for quantification of the lignin structures identified, and **Fig. 4** for the main lignin structures identified.

Table 5. Lignin units (S, G and S' molar percentages) and interunit linkages (side-chains per 100 units) from the HSQC spectra of eucalypt samples at two stages (2 and 8) of a multistage sequence, including 4 enzymatic treatments with *P. cinnabarinus* laccase and HBT and 4 alkaline peroxide extractions, compared with a control without enzyme and a treatment with laccase alone.

	Whole wood	
	stage 2	stage 8
1) Control		
- Lignin units (%):		
S (% S+G)	71	79
G (% S+G)	29	21
S' (% S)	2	5
S/G	2.4	3.8
- Lignin interunit linkages (%):		
β-*O*-4' Alkyl-aryl ethers (A) (% S+G)	39	43
Phenylcoumarans (B) (% S+G)	0	0
Resinols (C) (% S+G)	7	8
Spirodienones (F) (% S+G)	0	0
Total (% S+G)	46	50
2) *P. cinnabarinus* laccase		
- Lignin units (%):		
S (% S+G)	86	95
G (% S+G)	14	5
S' (% S)	4	28
S/G	6.2	17.5
- Lignin interunit linkages (%):		
β-*O*-4' Alkyl-aryl ethers (A) (% S+G)	26	31
Phenylcoumarans (B) (% S+G)	0	0
Resinols (C) (% S+G)	4	0
Spirodienones (F) (% S+G)	0	0
Total (% S+G)	30	31
3) *P. cinnabarinus* laccase-HBT		
- Lignin units (%):		
S (% S+G)	100	100
G (% S+G)	0	0
S' (% S)	29	62
S/G	----	----
- Lignin interunit linkages (%):		
β-*O*-4' Alkyl-aryl ethers (A) (% S+G)	19	14
Phenylcoumarans (B) (% S+G)	0	0
Resinols (C) (% S+G)	0	0
Spirodienones (F) (% S+G)	0	0
Total (% S+G)	19	14

3.3. Saccharification of wood pretreated with two laccase-mediator systems

In addition to studying in detail the structural modification of lignin from eucalypt wood during the pretreatment with two different laccase-mediator systems in a multistage sequence consisting of 4 cycles (8 stages), the effect of these treatments on lignin content and saccharification yields after enzymatic hydrolysis was studied. **Table 6** shows the lignin content (as Klason lignin) and glucose and xylose released after the enzymatic hydrolysis with cellulases of eucalypt samples pretreated with the *M. thermophila* and *P. cinnabarinus* laccases (data after first and last cycles of the sequence). The pretreatment of eucalypt wood with *M. thermophila*-MeS and *P. cinnabarinus*-HBT produced a decrease in lignin content after the 4 cycles of about 50% in both cases. This decrease in lignin content paralleled the increase in enzymatic saccharification yield of glucose of almost 30% with both pretreatments. Therefore, both laccase-mediator systems produced similar effects with regard to lignin content and glucose release after the four cycles of pretreatment. Surprisingly, the pretreatment with the two laccases alone produced different effects. Whereas the pretreatment of eucalypt wood with the low redox potential laccase from *M. thermophila* produced a decrease of lignin content of about 20%, the pretreatment with the high redox potential laccase from *P. cinnabarinus* did not affect the lignin content. Concerning glucose release, the treatments with *M. thermophila* and *P. cinnabarinus* laccases alone produced an increase of glucose release of 9% and 4%, respectively.

Unlike at the end of the sequence, considerable differences were observed between the two laccase-mediator treatments after the first cycle of pretreatment. While the pretreatment with *M. thermophila*-MeS did not produce any effect in lignin content (or glucose release), that with *P. cinnabarinus*-HBT decreased the lignin content in 14% and increase the glucose release by 8%. On the other hand, the lignin content and saccarification yield with both laccases alone were not modified after the first cycle of pretreatment.

Table 6. Lignin content (as Klason lignin) and monosaccharide release (both as % of sample weight) from cellulase hydrolysis of eucalypt samples from cycles 1 and 4 of a multistage sequence, including 4 enzymatic treatments with *M. thermophila*-MeS or *P. cinnabarinus* laccases-HBT and 4 alkaline peroxide extractions, compared with a control without enzyme, a treatment with laccase alone, and the initial eucalypt wood. Means ± S.D (from triplicates).

	Lignin (%)	Glucose (%)	Xylose (%)
1) Initial eucalypt wood	22.3 ± 0.3	39.5 ± 1.1	6.7 ± 0.1
2) *M. thermophila* laccase			
- 1 Cycle:			
Control	21.2 ± 0.8	39.7 ± 0.2	6.7 ± 0.1
Laccase	21.1 ± 0.9	39.5 ± 0.7	6.6 ± 0.2
Laccase-MeS	21.1 ± 0.8	39.7 ± 0.4	6.5 ± 0.3
- 4 Cycles:			
Control	21.1 ± 1.0	43.7 ± 0.2	7.5 ± 0.1
Laccase	16.8 ± 0.3	47.8 ± 1.2	8.1 ± 0.2
Laccase-MeS	11.2 ± 0.3	55.7 ± 0.4	9.1 ± 0.1
3) *P. cinnabarinus* laccase			
- 1 Cycle:			
Control	19.5 ± 0.9	39.6 ± 0.1	6.7 ± 0.0
Laccase	19.5 ± 0.9	39.9 ± 0.2	6.7 ± 0.0
Laccase-HBT	16.8 ± 0.9	42.9 ± 0.6	7.7 ± 0.1
- 4 Cycles:			
Control	17.9 ± 0.9	45.6 ± 0.7	8.3 ± 0.1
Laccase	17.9 ± 0.9	47.4 ± 0.7	8.2 ± 0.1
Laccase-HBT	9.6 ± 1.0	58.0 ± 0.2	7.6 ± 0.1

4. Discussion

Eucalypt is a rapidly growing and high biomass-producing tree used as a raw material for paper pulp manufacturing in several countries including Southwest Europe, Brazil, Uruguay and South Africa. Among the different eucalypt species, wood from *E. globulus* is the best raw material for kraft pulp manufacturing due to the high pulp yield [25]. Additionally, the lignin of *E. globulus* is enriched in S units with β-*O*-4' linkages predominating [25-27]. This implies linear chains with less cross-linking than G-rich lignin because of the methoxylated and thereby, blocked C-5 position in the syringyl unit, being advantageous for delignification purposes. Additionally, the higher occurrence of β-β units (resinols) observed in S-rich lignin [26] leads to shorter chain lengths and thus, lower molecular weights [28]. With all these characteristics, *E. globulus* wood has great potential as lignocellulosic feedstock for the production of second generation wood-based bioethanol [29]. However, as a lignocellulosic raw material, a pretreatment of eucalypt wood to overcome its recalcitrance towards enzymatic hydrolysis (mainly due to the presence of lignin) is required. After the first studies showing the potential of *M. thermophila*

laccase-MeS pretreatment, followed by alkaline peroxide extraction in a multistage sequence, to remove lignin from eucalypt (*Eucalyptus globulus*) feedstock improving saccharification after enzymatic hydrolysis [19], an interest arouse to deepen in the study of the modification of lignin structure along the complete sequence since lignin removal and its structure after pretreatment are crucial to promote fibre structure disruption and facilitate enzymatic hydrolysis.With this aim, samples of whole eucalypt material, their isolated lignins and filtrates at each stage of the whole sequence (8 stages) were characterized by 2D-NMR. Additionally, a comparison between this low redox potential laccase-mediator and a high redox potential laccase-mediator treatment using *P. cinnabarinus* laccase and HBT on delignification and saccharification yield was also performed. Finally, the relationships between lignin content and structure in the pretreated raw material and efficiency of the carbohydrate enzymatic saccharification has been targeted.

4.1. Eucalypt lignin modification by a commercial laccase and a natural mediator

The detailed study of lignin structure along the enzymatic sequence revealed that some of the main changes observed by 2D-NMR of the whole material after the complete sequence, namely the absence of G units and resinol substructures together with a high decrease in β-*O*-4' alkyl-aryl ethers, were already evidenced after 2 cycles of laccase-mediator treatment and alkaline extraction (stage 5). However, the most remarkable decreases in these units and substructures were observed indeed in the second enzymatic treatment (stage 3) together with the most remarkable increase in C_α-oxidized lignin units (S'). This modification of lignin structure was produced in the enzymatic stages. Even more, although the first cycle of pretreatment did not produce any decrease of the lignin content (as revealed by Klason lignin determination) the 2D-NMR results revealed modification of the lignin structure, already in the first enzymatic treatment (stage 1) including decrease in β-*O*-4' alkyl-aryl ethers, resinol substructures and increase in the S/G ratio. This indicates that the laccase-mediator treatment is able to modify lignin in a lignocellulosic material without a previous chemical pretreatment. The most remarkable effect observed during the alkaline peroxide stages was the decrease of C_α-oxidized lignin units (S') as well as acetylated xylan units (X´). The positive effect of the alkaline peroxide extraction following the enzymatic treatment with *M. thermophila* laccase and MeS was also observed during the delignification of eucalypt paper pulp [20]. The pretreatment with *M. thermophila* laccase alone, also produced a decrease in β-*O*-4' alkyl-aryl ethers and increase in C_α-oxidized lignin units although to a less extent that in the laccase-mediator treatment. Likewise, removal of resinol substructures also occurred

although in the last enzymatic treatment. The amount of G units decreased along the sequence but they were still present at the end of the pretreatment. The modification of lignin structure by the laccase alone, without mediator, suggests either the modification of the phenolic-type lignin (maybe enabling a progressive degradation of the polymer) [30] or the involvement of natural phenolic structures mediating the enzymatic oxidation.

The 2D-NMR analysis of CEL preparations corroborated the main findings observed in the pretreated whole feedstock compared with the controls, namely the increase in the S/G ratio and C_α-oxidized lignin units along the sequence, and the decrease in resinol substructures, especially evident in the treatment with laccase and mediator. On the other hand, when comparing the lignin composition of the filtrates obtained at different stages of the pretreatment with the corresponding residual lignin (CEL) it can be observed that in control samples, a similar relative composition of aromatic units and interunit linkages was observed in both lignins from stage 2 revealing that alkaline extraction in this stage extracted part of the lignin present in wood samples without altering its composition. However, in the stage 8 the lignin present in the filtrates lacked phenylcoumarans, resinols and spirodienones substructures whereas these substructures were present in the corresponding CEL preparations. Concerning lignins from laccase (alone) and laccase-mediator pretreatment, a relatively similar composition was also observed between lignins in filtrate and wood (CEL) from stage 2, including the proportion of lignin interunit linkages (per 100 phenylpropane units). However, a significant decrease in the number of side-chains involved in the different lignin substructures was observed in the filtrates of stage 8 compared with its corresponding CEL lignin indicating that lignin in the filtrate was more depolymerized than residual lignin in wood. In addition, a stronger increase in C_α-oxidized S units (S') from stage 2 to 8 in lignin from filtrates than in CEL lignin was observed. In CEL lignin the increase in S' was associated with an increase in β-*O*-4' ether linked C_α-oxidized side chains ($A_{ox\beta}$) whereas the contrary happened in lignin from filtrates. This suggests that these oxidized units in CEL lignin are linked while in filtrates may be not. On the other hand, the increase in S/G ratio observed along the sequence in both the whole samples and isolated lignin (CEL) was not paralleled by a predominance of G units in the filtrates. This may suggest that G units degraded by enzymatic treatment undergo either condensation reactions forming 5-5' or 4-*O*-5' linkages, or reactions leading to aromatic ring opening.

4.2. Comparison of lignin modification by *M. thermophila*/*P. cinnabarinus* treatments

For comparative purposes, the pretreatment of eucalypt wood was also carried out with the high redox potential laccase from the basidiomycete *P. cinnabarinus* and the mediator HBT, and the results compared with those obtained in the pretreatment with the low redox-potential laccase from the ascomycete *M. thermophila* and the phenolic mediator MeS. Although the decrease in lignin content after the whole sequence of the pretreatment of eucalypt wood with both laccase-mediator systems was similar (~50%), the structure of the residual lignin in eucalypt feedstock after both treatments showed some differences. Although in both cases the lignin composition in terms of aromatic units was the same since both lignins lacked G units after the treatments, the residual lignin of the *P. cinnabarinus* laccase and HBT treatment was more oxidized (higher amount of C_α-oxidized lignin units) and degraded (lower amount of β-*O*-4' alkyl-aryl ethers per 100 phenylpropane units) than lignin in the treatment with *M. thermophila* laccase-MeS. The differences in residual lignin structure (and content) with both treatments became more evident in the initial stages (cycle 1) of the treatment. At stage 2 the lignin from treatment with *P. cinnabarinus* laccase and HBT was only constituted by S units (some of them oxidized) and rather degraded whereas the lignin from the pretreatment with *M. thermophila* laccase-MeS was constituted by both S and G units (S/G ratio of 5.4), was less degraded (higher amount of β-*O*-4' alkyl-aryl ethers) and still contained high content of resinol substructures. Likewise, an initial decrease (14%) in lignin content was observed only in the treatment with *P. cinnabarinus* laccase. In a similar way, higher delignification of eucalypt pulp was attained in the treatment with *P. cinnabarinus* laccase and HBT compared with that using *M. thermophila* laccase and MeS [31]. Nevertheless, although at a different rate, wood lignin modification by laccase in the presence of MeS also yielded a structural modification pattern characterized by extensive C_α-oxidation (as shown by 2D-NMR) suggesting that the attack mechanism by laccase in the presence of MeS is the same reported by laccase-HBT. This agrees with results from model compounds showing that laccase in the presence of phenolic mediators oxidize non-phenolic aromatic compounds *via* a hydrogen abstraction mechanism [32].

The pretreatments of eucalypt feedstock with the two laccases in the absence of mediators already showed differences in lignin modification. At initial stages (step 2) no decrease in lignin content was observed with either *M. thermophila* or *P. cinnabarinus* laccases alone. However, whereas the lignin structure of wood from treatment with the former laccase was barely modified (with respect to the control), that from treatment with the latter laccase showed an increase in the S/G ratio compared with the control (from 2.4 to 6.2), an increase in C_α-oxidized lignin units and a decrease in the amount of β-*O*-4' alkyl-aryl ethers (per

100 phenylpropane units). Surprisingly, at the end of the sequence (stage 8) only *M. thermophila* laccase, and not the one from *P. cinnabarinus*, decreased the eucalypt lignin content (about 20%) when applied alone, in spite of the latter being a high-redox potential laccase unlike the former. A similar finding was reported in the treatment of eucalypt pulp with these two laccases [20]. In spite of this, the structure of residual lignin from the treatment with *P. cinnabarinus* laccase was more modified than that from *M. thermophila* laccase, being more oxidized and depolymerized and presenting a higher S/G ratio. A similar pattern of degradation, although with differences in reaction rates, has been reported in the reaction of lignin model compounds with two laccases differing in their redox potential [33].

4.3. Lignin modification by laccase-mediator and enzymatic saccharification yields

In addition to study the modification of lignin structure along the enzymatic pretreatment sequence with the two laccase-mediator systems, another goal of the present work was to study the relationships between lignin content and structure in the pretreated raw material and the efficiency of the enzymatic saccharification. First, it should be mentioned that some increase in sugar release (up to 10% and 15%) were observed in control samples after 4 cycles of pretreatment (not observed after one cycle) of both treatments with respect to initial wood. This could be related with the removal of acetylated xylan units (X´) produced by the alkaline extraction conditions. These units were still present in control samples after one cycle of pretreatment but not after 4 cycles. The effect of alkaline saponification of acetyl ester bonds in the improvement of enzymatic accessibility to the polysaccharides has been reported [34].

The delignification (~50%) produced at the end of the pretreatment by the two laccase-mediator systems correlated in both cases with similar (~30%) increases in glucose yield. Likewise, at the beginning of the sequence (stage 2), the pretreatment with *M. thermophila*-MeS did not decrease the eucalypt lignin content and consequently no increase in glucose release was observed, whereas an increase in glucose release (by 8%) was produced in the pretreatment with *P. cinnabarinus*-HBT where a decreased the lignin content (14%) was attained. This suggests a direct correlation between the decrease in lignin content and increase in enzymatic saccharification yield with the laccase-mediator treatments. This correlation is also observed in the pretreatments with laccases alone at the beginning of the sequence where no decrease in lignin content and no increase in enzymatic saccharification were produced. Different findings have been reported on the effects of wood lignin content on sugar release [2,35].

In addition to lignin content, lignin composition (S/G ratio) and structure also can influence enzymatic saccharification. As described above, the removal of guaiacyl lignin units was one of the main features observed during the pretreatment of eucalypt feedstock with the two different laccase-mediator systems. The presence of less guaiacyl units results in a less branched lignin structure, which may facilitate carbohydrates accessibility and hydrolysis [36]. This can explain why at the end of the sequence of the pretreatment with *P. cinnabarinus* laccase alone (stage 8) a slight increase in glucose release (about 4%) was produced without a decrease in lignin content. This increase in glucose release can be attributed to the modification of lignin structure, namely the strong increase in S/G ratio (from 3.8 to 17.5) besides a higher lignin degradation by *P. cinnabarinus* laccase pretreatment. However, the increase of S/G ratio (from 2.9 to 5.4) after one cycle of *M. thermophila*-MeS pretreatment did not increase the sugar release. This suggests that S/G ratio alone did not correlate with the amount of sugar released. Although it is generally understood that low lignin contents increase the ability of cellulolytic enzymes to hydrolyze plant fibers [35,37], no clear trend has been found on the effect of S/G ratio on sugar release [2,29,37]. It can be concluded that lignin content combined with lignin structure determines the efficiency of enzymatic hydrolysis. As lignin content decrease, the released sugars increase. If lignin content is the same (and not very high), a higher S/G ratio (by removal of guaiacyl units) promote a more accessible substrate and results in higher enzymatic hydrolysis efficiency.

5. Conclusions

This work shows further insights in the modification of lignin structure and content produced by two laccase-mediator pretreatments consisting of a sequence of successive laccase-mediator and alkaline extraction stages, directly on ground lignocellulosic material (i.e. without a previous chemical deconstruction) and its relationship with enzymatic saccharification yields. Both high and low redox potential laccase-mediator pretreatments delignified eucalypt wood (up to 50%) and improved (~30%) enzymatic sugar release after the complete multistage sequence, although differences on lignin modification/removal extents were observed at the several stages of the sequence. The HSQC NMR spectra of the lignocellulosic samples showed a significant decrease of both aromatic (with preference of G units) and aliphatic lignin signals after the enzymatic treatments, and provided strong evidence for a C_{α}-oxidation degradation mechanism, with high presence of oxidized S units in the residual lignin. The results obtained demonstrate that the reduction in lignin content effectively overcame cell wall recalcitrance to enzymatic

saccharification. However, lignin composition (S/G ratio) alone may not be a major factor affecting enzymatic hydrolysis, and changes in lignin structure most probably also cause a significant effect.

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La presente Tesis aborda el estudio de tratamientos enzimáticos (basados en la utilización de lacasas en presencia de mediadores redox) encaminados a eliminar/modificar la lignina y/o lípidos presentes en materiales lignocelulósicos de interés industrial, con el fin de conseguir un aprovechamiento más eficaz y racional de dichos materiales, tanto para la producción de celulosa como de biocombustibles de segunda generación (bioetanol). En particular, se ha utilizado el sistema lacasa-mediador tanto para la deslignificación/blanqueo y eliminación simultánea de lípidos en pastas de celulosa (pastas kraft de eucalipto), como para la deslignificación de materiales lignocelulósicos (madera de eucalipto y hierba elefante). Las principales conclusiones obtenidas se citan a continuación:

1. El tratamiento enzimático de las pastas de eucalipto con lacasa de *Myceliophtora thermophila* y los mediadores naturales siringaldehído y siringato de metilo (seguido de extracción alcalina con peróxido de hidrógeno), mostró una gran eficacia para eliminar tanto la lignina residual de la pasta como los lípidos responsables de los problemas de *pitch*, obteniéndose mejores resultados con el siringato de metilo en cuanto a deslignificación (25%) y blancura (aumento de 8 puntos de blancura ISO) de las pastas y con el siringaldehído en la eliminación de los lípidos (73% de esteroles libres, 91% de esterol glicósidos y 89% de ésteres de esteroles).
2. Los efectos positivos del tratamiento anterior sobre la pasta de eucalipto, especialmente en lo referente a la blancura, sólo se observaron tras la extracción alcalina con peróxido, revelando estos resultados la necesidad de una extracción alcalina con peróxido en una secuencia de blanqueo enzimática que utilice lacasa y mediadores naturales.
3. El pretratamiento con lacasa de *Trametes villosa* y el mediador sintético HBT mostró una gran eficacia para deslignificar la madera de eucalipto (48%) y hierba elefante (32%) así como para mejorar el rendimiento de la sacarificación enzimática de ambos materiales lignocelulósicos (aumento de un 61% y 12%, en el rendimiento de glucosa, respectivamente).
4. El pretratamiento con lacasa (comercial) de *Myceliophthora thermophila* y el mediador fenólico siringato de metilo resulta una alternativa prometedora al tratamiento anterior dando lugar a una deslignificación de un 50% de la madera de eucalipto y a una mejora en la sacarificación enzimática (30% aumento en la liberación de glucosa) con respecto a la madera de eucalipto sin pretratamiento enzimático.

5. Los análisis de 2D-NMR de las muestras de eucalipto y hierba elefante tras los pretratamientos enzimáticos con los sistemas lacasa-mediador utilizados en esta Tesis, revelaron en todos los casos, una eliminación de las unidades guayacilo y siringilo de la lignina con preferencia de las primeras, una oxidación de la lignina evidenciada por el aumento de las unidades de siringilo oxidadas en el C_α y una disminución/desaparición de los enlaces β-*O*-4′.

En conclusión, los tratamientos enzimáticos de materiales lignocelulósicos, basados en el sistema lacasa-mediador presentan un gran potencial tanto para la deslignificación/blanqueo de pastas de celulosa como para la obtención de bioetanol de segunda generación.